日本癌学会及財團法人癌研究会編集発行

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"GANN"

THE JAPANESE JOURNAL OF CANCER RESEARCH

Founded by K. YAMAGIWA and Continued by M. NAGAYO

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[GANN, Vol. 46; March, 1955]

HYPERPLASTIC LESIONS OF THE GLANDULAR STOMACH OF RATS BY ORAL ADMINISTRATION OF 20-METHYLCHOLANTHRENE (With Plates I-VI)

KAZUO MORI, ICHIRO HIRAFUKU, TADASHIGE MURAKAMI and SHOGO ICHII

(Showa Medical School, Tokyo)

Adenocarcinoma of the glandular lesions of the stomach has not been induced by feeding the carcinogenic hydrocarbons to mice and rats, although carcinoma of the forestomach and/or of the small intestine may occur (1-5). And these efforts were reviewed by Klein and Palmer and Sugiura (6, 7). However, the glandular mucosa is susceptible to the action of carcinogenic agents when introduced directly into the wall of the stomach (8-15).

The induction of the hyperplastic lesions including the adenomatous growths in the glandular stomach of rats receiving emulsions containing 20-methylcholanthrene was investigated in our laboratories. And the results are reported in the following notes.

Materials and Methods

235 male rats of the Wistar strain and mixed stock, 1 to 4 months of age and weighing 45-125 g were employed. They were fed on the rice diet, containing of 2 per cent of anhydrous sodium carbonate, 2 per cent of fish powder and a small amount of cod liver oil in polished rice. The hydrocarbon was administered in an aqueous emulsion of 4-10 per cent polyethylene glycol. In preparing emulsion, 500 ml of polyethylene glycol was heated over a water bath and 0.5 g of 20-methylcholanthrene (Eastman Kodak Co. and L. Light Co.) was dissolved. 5 to 10 ml of this solution was mixed with 100 ml of tap water in the bottle. The bottle has a wide mouth and a rubber stopper, into which was inserted a glass tube 8 mm wide and 10 cm long which passes perpendicularly through the top of a wooden box, almost similar to that described by Lorenz and Stewart (4). The average daily intake of emulsion and hydrocarbon per rat was calculated from the total consumption of the group. The consumption per rat per day fluctuated considerably, as the average amount of the hydrocarbon ingested varied from 0.2 to 1.0 mg or more per day.

20 control animals were maintained on the diet described above and received the polyethylene glycol emulsion without the carcinogenic hydrocarbon.

Food and fluid intake were not restricted in all groups. And green vegetables

were occasionally supplied. Though diet and drink were not discontinued until the end of the experiment, the loss of weight per animal was great. Animals which appeared to be dying were killed by cervical dislocation, and the stomachs were removed and placed in a bottle of fixative. After the slight fixation, the stomachs were sectioned in a plane passing through both curvatures. The mucosal surface was inspected carefully and appropriate sections excised. The tissues were embeded in paraffin and cut and stained routinely with hematoxylin and eosin. Occasionally, silver impregnation methods for reticulum were employed. In most cases, serial sections were prepared from appropriate specimens.

Results

Healthy young rats placed on the diet and the emulsion of the carcinogen deteriorated rapidly due to diarrhoea and then animals seemed to thrive less than normal. They showed loss of vigor with dull and lusterless coats. Therefore it was difficult to keep the experimental animals alive for a long period, and great number of them died during the first one to two months of the experiment as shown in Table 1, before the effects of the methylcholanthrene became manifest.

Table 1.
Adenomatous Growths in the Glandular Stomach Induced by Methylcholanthrene Emulsion.

Group	Strain of rats	Number of rats at biginning	Average of initial body weight of rats	Age of rat at beginning of experiment	Number of rats survived more than 2 months	Number of rats with adenomatous growths (survival days)
1	Mixed stock	15	125 g	4 months	9	none
2	〃	20	45	2	7	1 (68)
3	〃	50	70	2.5	17	none
4	〃	50	65	2.5	10	none
5	Wistar strain	20	65	2.5	8	3 (113, 122, 123)
6	〃	30	60	2	6	none
7	〃	50	45	1	7	1 (80)
Total		235			64	5

The glandular stomach of rats receiving the emulsions containing methylcholanthrene was usually edematous and rough. This altered appearance may be often noticed in the pyloric antrum of the stomach. Grossly, there was little to differentiate the early change of gastric lesion from the normal (Fig. 1). But marked hyperplastic lesions present distinctive features (Figs. 2-6). To the naked eye these lesions appear as irregular and raised edges in the mucosa of the stomach after it had been opened. In the pronounced cases, the lesions

are more tortuous or labyrinthine and there was a variable degree of excessive foldings (Plate 1, Fig. 6). These lesions were greyish white in color except when they showed congestion, hemorrhage, slight necrosis or secondary infection. 5 adenomatous growths occurred out of 64 effective total of animals which survived more than two months. But, no adhesion extending between other organs was found. The periods of time between the beginning of the administration of methylcholanthrene orally and the finding of the marked hyperplastic lesions of the glandular stomach at autopsy was as follows for the rats of the different experimental groups: Group II–68 days: Group V–113, 122 and 123 days: Group VII–80 days. Then the range was from 68 to 123 days, and the corresponding amount of an average ingestion of hydrocarbon was 30 to 100 mg approximately.

It is of special interest to note that no suspicious lesions appeared in the proventriculus of these rats in this experiment. In few animals, however, the limiting ridge was slightly prominent and somewhat beaded.

There was also a variation in the number of the gastric lesions induced depending upon the strain of rat employed. Wistar strain is susceptible and the mixed stock rat was refractory under this experimental condition.

Pathological Observations

The pathological changes in the glandular stomach of the experimental rats are identified into the following four groups: 1) the various grades of the erosion in the mucosa (Fig. 7), 2) the tubular elongation of the crypts of the glands (Fig. 12), 3) the epithelial-lined excavations in the mucosa (Fig. 8) and 4) the adenomatous proliferation of the atypical epithelial cells within the wall of the stomach (Figs. 9–17).

As the early changes preceding the formation of these hyperplastic lesions, a few pathological lesions were found. The mucosa was destroyed, and the epithelial excavations were formed. Some part of these new epithelial cells first relined the excavation often transformed into atypical epithelial cells and then proliferated to form an adenomatous growth (Fig. 9). The morphology of this atypical epithelium is not similar the acid-pepsin secreting epithelium of its origin (Figs. 10 and 11). The complete histological description of the early lesions will be reported in another publication because they may have close relation to the histogenesis of the stomach cancer.

With the passage of time, the atypical cells of the new epithelium extended and replaced the covering epithelium (Fig. 12), and often invaded through the muscularis mucosa into the submucosa in the form of cords, nests and acini (Figs. 13 and 14). In some cases these growths extended nearly to the serosa through the submucosa and the muscular coat (Figs. 14 and 15). Some of them became irregular in size and shape and mitotic figures became more numerous

but not bizarre. There was a moderate variation in the appearance of the glands composing the adenomatous and lumenizing and occasionally solid growths (Figs. 16 and 17) and the cells lining them were frequently hyperplastic, hyperchromatic and relatively large in appearance. Many of the cells were cuboidal and columnar in shape, and the cytoplasmic vacuoles seen in hematoxylin and eosin preparations were easily demonstrated to contain mucus by the use of mucicarmin staining. However, in all specimens, the cellular elements of these growths did not exhibit the disordered cellular pattern.

In this experiment, young animals were more susceptible to the induction of these gastric lesions than old.

It is impossible to state at this time, whether these adenomatous growths regress, or remain long as they are, or ultimately develop into gastric carcinoma.

The evidence of systemic effects, such as severe atrophy of the genital organs, the spleen, lymph nodes, hematopoetic tissues and the lesions of the lung were detected and the complete pathological description of these lesions will be reported in another publication.

In control animals, no remarkable change was detected both in the proventricular and glandular portion of the stomach.

Discussion

Many investigators have shown repeatedly over the past ten years that the glandular stomach of rats and mice completely resisted carcinomatous transformation when various carcinogenic hydrocarbons were administered in drinking-water emulsions (16–19). Stewart and Lorenz have shown, however, that when methylcholanthrene suspended in horse serum is injected into the wall of the prepyloric stomach submucosally, adenocarcinomas resulted (20–23). On the other hand, it is reported also that when the carcinogens had been administered orally, squamous-cell carcinomas were produced in the forestomach of rats and mice. Namely, the labile nature of the squamous forestomach of animals hinders sound elevation of results in the glandular stomach (24). In 1945, Ivy discussed the problem of the function of the stomach as related to the development of cancer of the stomach and pointed out the possibility of a protective mechanism in the gastric mucosa (25). Under certain experimental conditions, e.g. hypochlorhydria or achlorhydria, we have come to assume that the glandular elements of the stomach of rats and mice would be affected by the methylcholanthrene directly, and then gastric absorption of the carcinogen would be possible (26).

In this experiment, the rats were maintained on the rice diet containing 2 per cent of sodium carbonate, 2 per cent of fish powder and a small amount of cod liver oil and were administered the emulsion of the methylcholanthrene in the polyethylene glycol as described above. This procedure of the rice diet

containing sodium carbonate was different from that of the previous investigators who failed to induce the lesions of the glandular stomach by oral administration of the carcinogenic hydrocarbons. In this experiment, the various grades of the erosion in the mucosa, the tubular elongation of the crypts of the glands and the formation of the excavations are observed as the early changes of the stomach wall preceding the adenomatous growths. Only 5 adenomatous growths occurred in 64 effective total of animals which survived more than two months from the beginning of the experiment. The range of the period was from 68 to 123 days. In one group (V), there was 3 adenomatous lesions (37.5%) out of 8 effective number of rats of Wistar strain. These adenomatous growths resemble in character "the adenomatous diverticulum" which is described by Hare et al. in rats after the intramural injection of the carcinogen into the stomach wall (15).

It is believed that the mucin layer which covers the whole glandular mucosa usually prevents the carcinogen from coming into direct contact with the stomach wall. Since the direct contact is the first condition for the absorption of the carcinogenic hydrocarbon, it is obvious that sodium carbonate in the rice diet might neutralize the gastric acid to make the stomach hypochlorhydric or achlorhydric, followed by the breakage of the mucus barrier and might make such a contact possible. From the physicochemical point of view, it is clear that the polyethylene glycol containing methylcholanthrene is able to contact with the stomach wall as demonstrated by Ermala et al. (27, 28). The carcinogen may penetrate into the mucosa of the glandular stomach along with the polyethylene glycol after the denudation of the mucosal surface under the presence of sodium carbonate. And by this means the carcinogen may work its way into the wall of the stomach enough to produce adenomatous growths described above, but may not be sufficient to produce cancerous change in the stomach tissue within the period covered by our studies.

In this case, the amount of the methylcholanthrene consumed fluctuated from 0.2 to 1.0 mg per rat per day, and it seems lesser than those of the previous investigators. However, when the concentration of the polyethylene glycol in which the methylcholanthrene was dissolved was raised the rats refused to drink the emulsion perhaps from its bitterness, and the emulsion often became creamy out of the fine crystals of the carcinogen. The longest period of the time of the experiment was 223 days, which we felt was too short when compared with that of previous workers whose experiments were usually conducted for over one year.

Within the limit of this investigation it has not proved that the adenomatous proliferation is precancerous in the sense that it is a lesion which may be occasionally followed by overt carcinoma. Investigations, planned along the

principles presented above, are now in progress.

Summary

235 male rats of the Wistar strain and the mixed stock were maintained on the rice diet containing sodium carbonate, fish powder and cod liver oil and 20-methylcholanthrene emulsion in polyethylene glycol administered orally, in drinking water for as long as 223 days. The morphological changes in the antrum of the glandular stomach of rats, such as the erosion of the mucus membrane, the tubular elongation of the crypt and the excavation lined with the new-epithelium with eventuated in the adenomatous growths of the glandular stomach are described. The majority of the adenomatous growths were composed of atypical glandular cells derived from the mucosa. These gastric lesions appeared to be confined to the chamber of the glandular stomach, and no remarkable change resulted in the part of the forestomach. However, no carcinoma was produced. At the present moment, it is an open question whether these adenomatous growths are precancerous, preceding the overt adenocarcinoma.

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要　旨

メチルコランスレン経口投与による白鼠腺胃腫様過形成について

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(昭和医科大学)

白鼠あるいは廿日鼠の腺胃に実験的胃癌を生成しようとする試みは，過去数十年に亘る主として米英諸研究者の不断の努力にも拘らず，いずれも不成功に終っている。これは経口的に与えられた癌原物質と腺胃上皮との接触が粘液の介在によって妨げられることと，前胃上皮がこれらの物質に対し顕著な感受性を有する結果として腺癌に先立って扁平上皮癌が生成されるためと考えられている。

本実験においては米に炭酸ソーダ・魚粉並に肝油を添加した飼料で動物を飼い，ポリエチレングライコールを溶媒としてメチルコランスレンを水に混じて与えることによって白鼠の腺胃に種々の増殖性変化を惹起することができた。

その変化の主なるものは 1) 種々の深さの糜爛，2) 胃小窩上皮の伸長，3) 再生した上皮で覆われた裂れ目，4) 種々の異型細胞，特に腺腫様過形成を示す細胞群の出現および増殖であった。これらの異型細胞のあるものは，ときとして配列が極度に乱れ，腺腔を失って充実性となり更に基底膜の著しい乱れを伴っていた。また時には粘膜筋層を貫いて粘膜下組織に達し，最も甚しい例では胃固有筋層を貫き漿膜に達せんとしている場合もあった。

このような異型細胞群は将来退縮してしまうのか，そのままの姿で止まるのか，あるいは所謂腺癌に先行するのか本実験の範囲では決定できなかった。　(文部省科学研究費による)

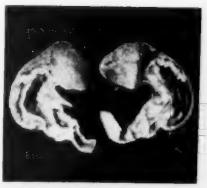


Fig. 1

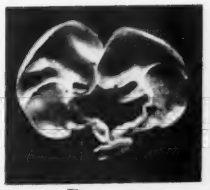


Fig. 2

Fig. 3

Fig. 4

Fig. 5

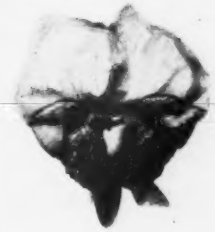


Fig. 6

Figs. 1–4 are the hemisections of the stomachs of rats which received methylcholanthrene orally.

Fig. 1. Death 60 days. The surface of the glandular stomach is smooth and vacant.

Fig. 2. Death 80 days. Note the irregular protuberances in the antrum, and the limiting ridge is beaded.

Fig. 3. Death 68 days. A small scar is seen on the surface of the glandular stomach. The wall of the antral portion of the both curvatures thickened by atypical growths.

Fig. 4. Death 123 days. Warty protuberances are seen in the antrum.

Figs. 5. and 6. Death 122 days. External (arrow) (5) and internal (6) view of the stomach of rat. Half part of the glandular stomach is replaced with the irregular and labyrinthine growths.

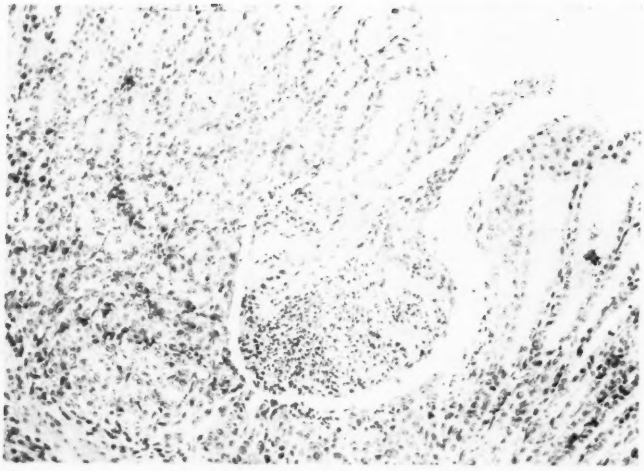


Fig. 7. Glandular mucosa of rat stomach. Note depth of cellular desquamation with the destruction of crypts (rat died on 60th day).

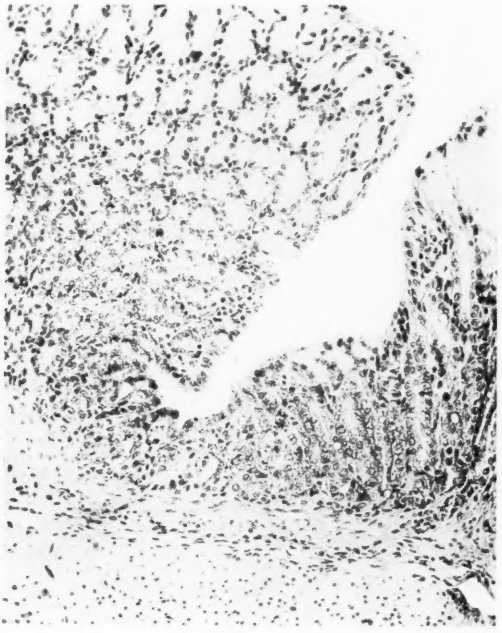


Fig. 8. Epithelial-lined excavation in the mucosa (rat died on 60th day).

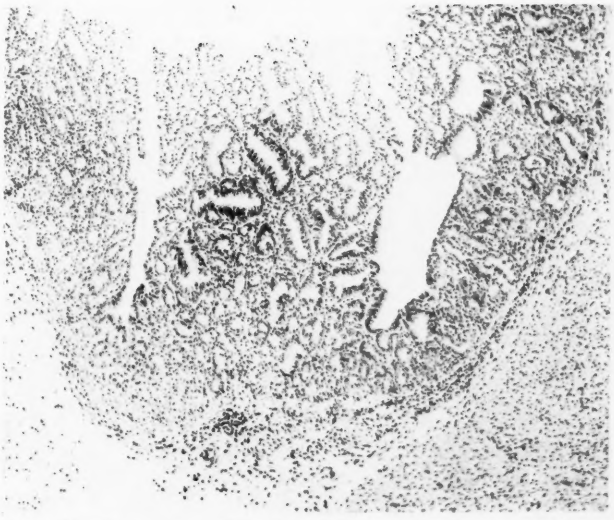
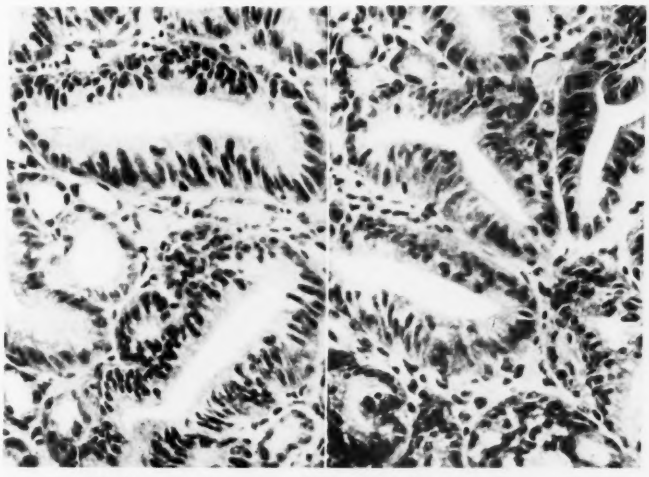


Fig. 9. Antrum (duodenum on right) of rats died on 68th day. Adenomatous growths of the atypical epithelial cells within the wall of the stomach.



Figs. 10 and 11. Higher power views of the area shown in Figure 9. Note irregular size and shape of the glands and numerous mitotic figures.

Fig. 12. One area of the glandular portion of the stomach, to demonstrate the early growth of the atypical epithelial cells (rat died on 68 th day).

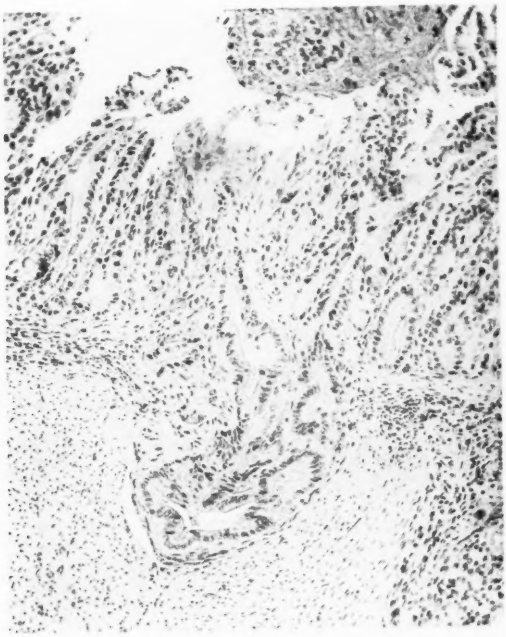


Fig. 13. Area shows atypical epithelial cells invading beneath the muscularis mucosa into the muscularis propria.

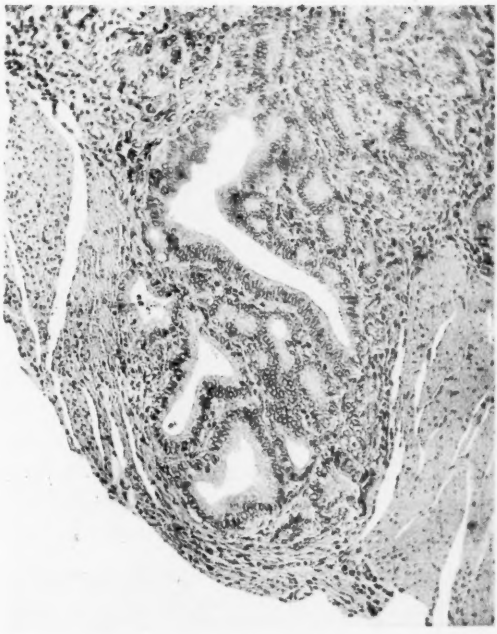


Fig. 14. Area shows more advanced features than in Fig. 13. Through muscularis mucosae and submucosa, atypical gland has extended into the muscular coat of the antrum of rat died on 122nd day.

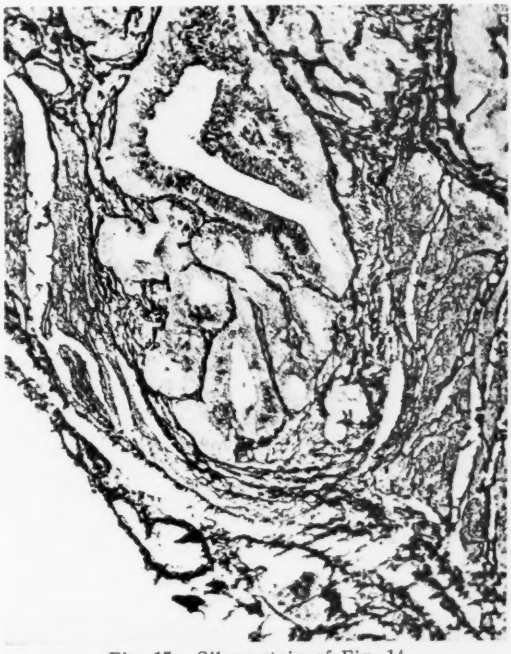


Fig. 15. Silver stain of Fig. 14.

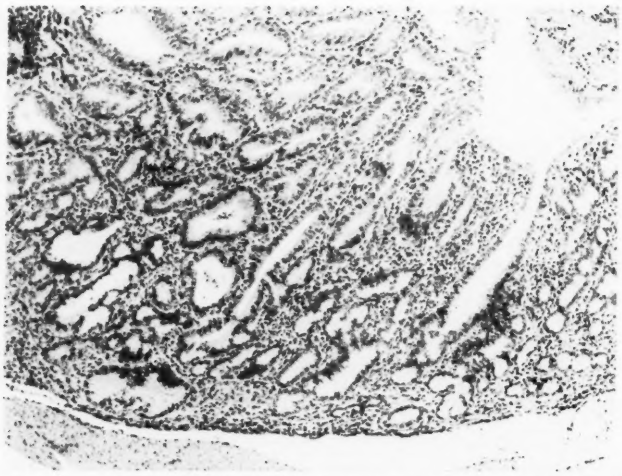


Fig. 16. The adenomatous growth of the atypical glands of rat died on 122nd day.

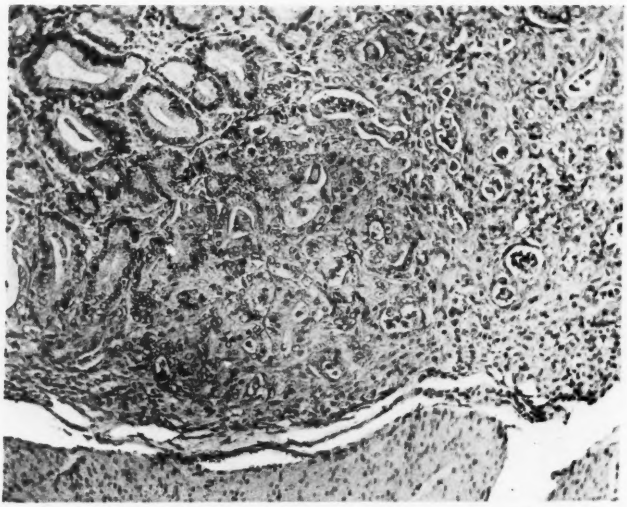


Fig. 17. The adenomatous area showing the more solid growth.

[GANN, Vol. 46; March, 1955]

A HISTOLOGICAL STUDY ON THE MECHANISM OF INTESTINAL EPITHELIAL METAPLASIA IN GASTRIC MUCOUS MEMBRANE
(With Plates VII and VIII)

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The authors are constantly endeavouring to clarify the histological development of gastric carcinoma with the materials obtained at their surgery. The authors in their study[1] of carcinoma solidum of the stomach have already reported that they have detected the developmental points of carcinoma cells and that they are found in a multicentric form. Although the developmental point of carcinomatous glandular lumina in adenocarcinoma has also been pointed out in another report,[2] the explanation of this developmental mechanism has been rather vague as compared to carcinoma solidum.

The reasons for this vague explanation are considered as follows: 1) The developmental point in adenocarcinoma is not so clear-cut, as in carcinoma solidum. 2) The diagnosis of adenocarcinoma in the very early stage is very difficult. In order to overcome these obstacles, the authors selected a round about way; efforts were made to obtain a more reliable clue in deciding the developmental form of adenocarcinoma first by clarifying the developmental mechanism of intestinal epithelial cells which is said to appear as metaplasia[3] in the gastric mucous membrane, and then by its comparison with adenocarcinoma.

Although the intestinal epithelium is hardly observed in the gastric mucous membrane of young persons, it is nearly always observed in the stomach of old persons and in cases of chronic gastritis. This is considered as one of the chief findings in "concomitant gastritis" which is found in gastric carcinoma.[4] Therefore, one is apt to hold a question as to whether there is some interrelation between the developmental course of both carcinoma and intestinal epitelium. If there is anything similar in their causal development, one may presuppose that there is also a similar relationship between their morphological development, but it may be stated that no sufficient comparative studies have been made between these two processes.[3)5)6)]

In considering the above conditions, one is led to believe that this way of thinking is not a detour but rather a promising trial in comparing and examinig two important denaturalizations such as the development of carcinoma and in-

testinal epithelial metaplasia. In this report the authors will discuss chiefly the histological development of intestinal epithelial cells, and plan to report on the histological development of adenocarcinoma and its comparison with metaplasia later.

Case S. 34. T. Y. 43 years old woman. Clinical diagnosis, gastric ulcer. Received gastrectomy (Billroth II) on the 22nd of Sept., 1952.

The mucous surface of the removed stomach was immediately opened and fixed in Helly's solution. A small ulcer was found on the smaller curvature of the gastric angle. Surrounding this a reddened erosion covering an area of 3× 1.5 cm was noticed (Fig. 1). Eleven paraffin blocks were made from this lesion serially and serial sections were taken from each block, which added up to 7000 sections of 5 μ in thickness. In most of the sections, groups of atypical glandular lumina were observed here and there, and although their irregular structure led one to consider carcinoma, the authors hesitated in making this diagnosis since the change remained within the limits of the tunica mucosa and did not pentrate into the submucous tissue and, furthermore, no unusual marked proliferation of these cells could be proven histologically.

On the other hand, numerous groups of intestinal epithelial cells were observed in about the same surface as the groups of atypical glandular lumina above mentioned. It was confirmed by serial sections that most of the groups of intestinal epithelial cells have absolutely no connection with the adjacent group of intestinal epitelial cells and they are independent cubically. These independent groups of cells will be called intestinal epithelial islands.

The islands vary in size. Typical islands are shown in Figs. 2 and 3. The following figures including 2 and 3 all indicate the maximal cut-surface of each island. In investigating the morphological development of these intestinal epithelial islands, one will gradually search for those with a smaller cut-surface, which are as shown in Figs. 5–10.

Fig. 8 is the magnified picture of Fig. 7, and Fig. 10 treated with Schiff's reagent is the adjacent section to Fig. 9 stained with Hematoxylin-Eosin. From these stainings one may understand that the very small island in Fig. 9 is furnished with cuticular border, had one goblet cell and although small it has an entirely different character as compared to its surrounding foveolar epithelium. This was the smallest intestinal epithelial island observed. The width of its maximal cut-surface was 50 μ and the number of cells counted 7. The thickness of this island measured up to 60 μ (12 sections each 5 μ) and is composed of a group of numerous intestinal epithelial cells.

Investigating the size of the intestinal epithelial islands inversely from Fig. 10 to Fig. 2, one may presuppose that a small island such as shown in Fig. 9 gradually proliferated and developed into such an island as Fig. 7 to 2. It is

considered that a group of intestinal epithelial cells proliferate and replace the already existing epithelial cells in the surrounding. It can be understood that the mechanism of replacement constantly continues since a part of the unreplaced pseudopyloric glandular lumina (Diagram 1) can be observed in Fig. 4, which is a magnification of the basal portion of Fig. 3. Similar findings can also be readily observed in other intestinal epithelial islands. Oota[3] has already pointed out that not only intestinal epithelium but also other kinds of gastric epithelial metaplasia have tendency to progress chiefly by replacement.

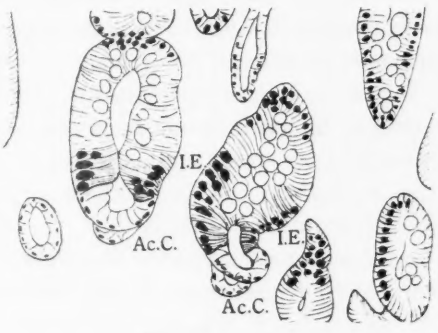
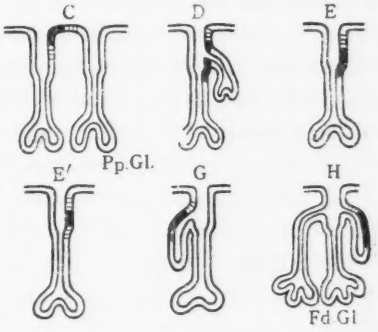


Diagram 1



Dirgram 2

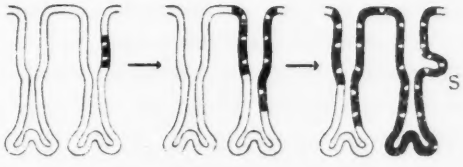


Diagram 3

Diagram 1 Explanation of Fig. 4. This is considered as one phase of the progressing replacement. I.E. stands for intestinal epithelium and Ac.C. for accessory cell before replacement.

Diagram 2 Six smallest intestinal epithelial islands detected in this case. Islands D, E, G & H. are adjacent to indifferent cells in the deeper layer (demonstrated by crossed stripes). Pp. Gl. stands for pseudopyloric gland and Fd. Gl. for fundus gland.

Diagram 3. Diagram of the proliferation from Fig. 10 to Fig. 2. This type of proliferation will be called development of the intestinal epithelial type. But no final decision could be made on the existence of a new branch S.

As to the problem of how an ialand such as shown in Fig. 10 develops, the authors can only rely on induction since no smaller islands could be detected. Although this island consists of numerous groups of cells as stated above, it can be presumed that at moment of development it consists of only one or a few cells.

In all, six intestinal epithelial islands with the same size as in Fig. 10 were observed in this case. These are shown in Diagram 2, and Fig. 10 corresponds

to E in the Diagram. Four out of six islands were found at the neck of the grstric glands, that is at the bottom of so-called gastric foveol, remaining one (H) situated a little deeper and the last one (C) at the surface of the mucous membrane. Each of the islands is chiefly surrounded by foveolar epithelium, but in 4 out of the 6 the cells lining the deeper part of the intestinal epithelial islands show a tendency of indifferent cells. These are shown in Diagram 2 in cross-stripes. Therefore, it is considered that the mother cells in which metaplasia occurred are chiefly the indifferent cells. Of course, one must be careful in considering the indifferent cells at the neck of the gland to be the only mother cells of metaplasia, because there is still left a question as to whether the regeneration epithelium repairing the mucous defect and the ordinary foveolar epithelium also have such an ability.[6] But, at any rate, it is noteworthy that the mechanism of intestinal epithelial metaplasia occurs in the same cell layer as the mother cells and develops by replacement.[3] The Diagram 3 is a model of the morphological development of the metaplasia, and the authors should like to call this form of development as "intestinal epithelial type."

The intestinal epithelial islands of Figs. 5–10 cannot simply be considered to develop into those of Figs. 2 and 3 only by the mechanism of replacement. There is a great possibility that 2 or more adjacent islands fuse together in forming such a large intestinal epithelial island. Furthermore one must also consider the possibility of the new formation of glandular lumia of intestinal epithelial cells. For instance, the glandular lumen "S" of intestinal epithlial cells in Fig. 11 is probably a newly formed lumen. Actually it is easy to find such a site of replacement as in Fig. 4, but on the other hand, it was very difficult in detecting a picture such as in Fig. 11, and the authors failed to find a similar picture in other sections. Therefore, although the mechanism of newly growing lumina cannot be denied in the development of intestinal epithelial islands, it cannot be considered to play a main role.

Among the numerous atypical glandular lumina in this case, there is a group of glandular lumina, which are similar to the intestinal epithelia, but more basophilic with fewer goblet cells, ill-defined cuticular border and with a basement membrane not closely packed (Fig. 12). In comparing this figure with Fig. 6, one may notice a close similarity, and this type of epithelium is considered to take a developmental form such as the "intestinal epithelium type." Since this type of epithelium has not only a remarkable atypical character but also a sporadic irregular structure, and pictures of extremely irregular lumina developing from themselves are observed, there seems to be near interrelationship to the development of carcinoma. This problem will be taken up in another paper.

Conclusion

Before deciding the histological development of adenocarcinoma, one must consider the development of intestinal epithelium. Serial sections were made from the surrounding of an ulcer obtained at surgery and numerous intestinal epithelial islands were observed. When the islands were arranged according to their size, one noticed that the intestinal epithelial cells developed *in situ* chiefly at the neck of the gastric glands, and they gradually developed towards the surrounding by replacement. The indifferent cells were considered to be their mother cells. Although the new formation of glandular lumina of intestinal epithelium cannot be denied in this case, it cannot be considered to play a main role. The existence of an atypical cells resembling intestinal epithelium and having the same developmental form as the intestinal epithelium was demonstrated.

Explanation of Plates

Fig. 1. Case S. 34. In the upper portion of the lesser curvature, a small ulcer can be observed with erosin in the surrounding. Redness was remarkable.

Fig. 2. The arrows indicate 2 intestinal epithelial islands.

Fig. 3. A higher magnification of the intestinal epithelial island found in the left of Fig. 2.

Fig. 4. A higher magnification of 2 glandular lumina found in the left of Fig. 3. Refer to diagram 1.

Fig. 5. A solitary group of glandular lumina of intestinal epithelium.

Fig. 6. A solitary glandular lumina of intestinal epithelium.

Fig. 7. A group of intestinal epithelial cells (arrow) found at the neck of a pseudpyloric gland.

Fig. 8. A higher magnification of Fig. 7.

Fig. 9. The smallest intestinal epithelial island found near the neck of a pseudopyloric gland. A higher magnification.

Fig. 10. A section adjacent to Fig. 9 treated with McManus stain. Cuticular border and atypy from adjacent cells are observed.

Fig. 11. Glandular lumina "S" may be considered as a newly growing lumina from its arrangement.

Fig. 12. A solitary glandular lumina of atypical cells. Compare with Fig. 6.

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要　旨

胃粘膜における腸上皮化生機転の組織学的研究

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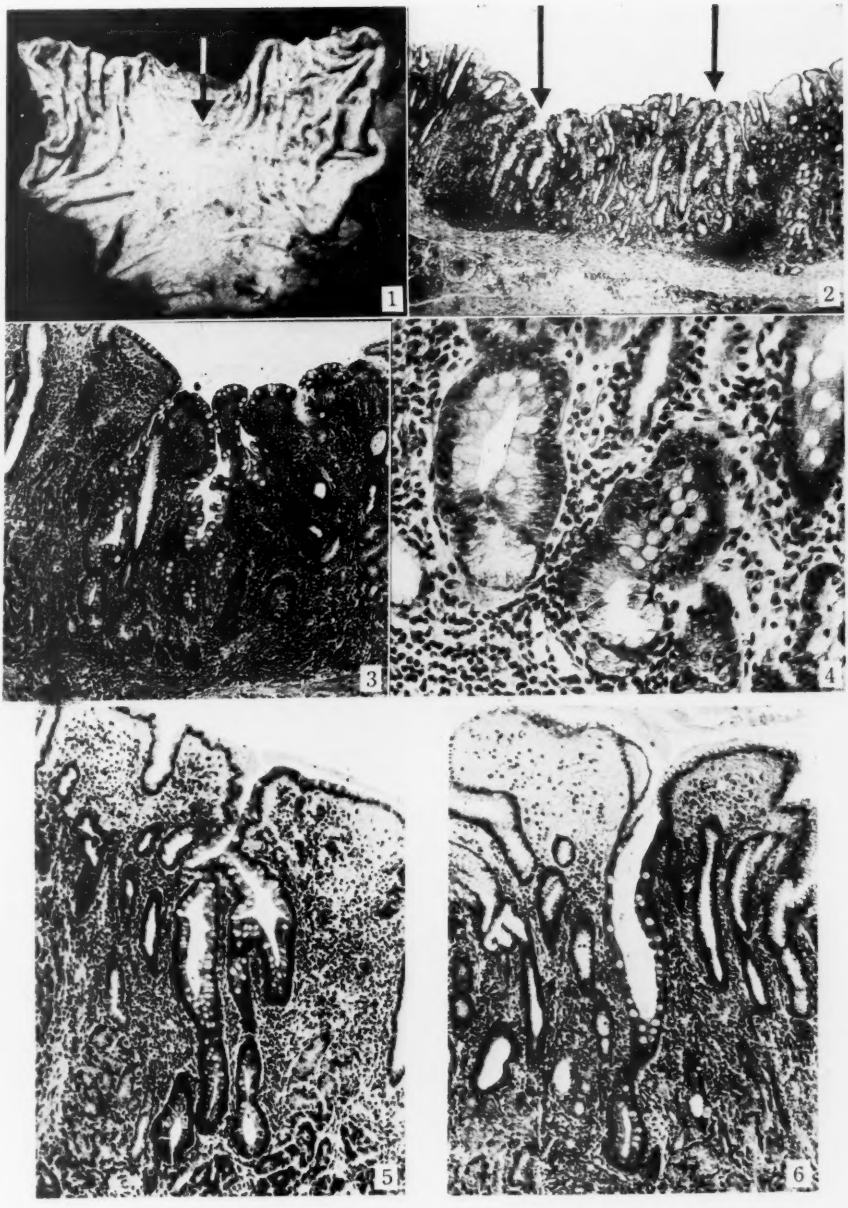
(昭和医科大学外科教室)

著者等は胃癌の組織発生を研究しつつあるが，充実癌に比し腺癌の発生型態が漠然としているのに悩んでいる。この困難をのりこえるために，胃粘膜に発生する腸上皮化生の機転を明かにし，それとの比較から腺癌発生のそれに関し，手懸りを得たいと望むようになった。著者等の外科で切除された胃潰瘍の中に，潰瘍周辺に奇妙な糜爛が存在する1例があった。そこから多数の連続切片を作製した所，多数の異型性腺腔群と腸上皮細胞群とが混然として発生しているのが見出された。腸上皮細胞群の中，連続切片によってその独立性が立体的に証明されたものを腸上皮細胞島と呼ぶことにし，それを大きさの順に並べると，腸上皮細胞島は主として胃小窩の最深部に当る不偏細胞帯に発生し，周囲の細胞を置換することによって次第に増殖拡大するものであると推論することが出来た。見出された最小の腸上皮細胞島は幅 50 μ，(細胞数7個)，厚さ 60 μ，で数十個の細胞群よりなっていた。かかる大きさの島が本例に6個あった。腸上皮細胞性腺腔の新生という問題は否定も出来ないが，腸上皮島の増殖には大きな役割を演じていないと考えられた。かかる型の新しい細胞群の発生形式を腸上皮型発生と呼びたい。

腸上皮細胞の発生(化生)母細胞は一応不偏細胞と考えられたが，一般の胃小窩上皮，再生上皮等にこの能力があるかどうか，等の問題は未決定である。

また腸上皮細胞に似て非な異型性細胞群が本例には多数みられ，かつそれらが，腸上皮島との構造の比較から腸上皮型発生型式をとることも推定された。それらの異型細胞群はそれ自身異型性が強いのみならず，それらから構造の乱れ，染色性の変化等の強い細胞群が発生している像が見出されるので，その悪性度如何ということは極めて重要な問題であると思われるがこれについては別の機会に論ずるつもりである。

(文部省科学研究費による)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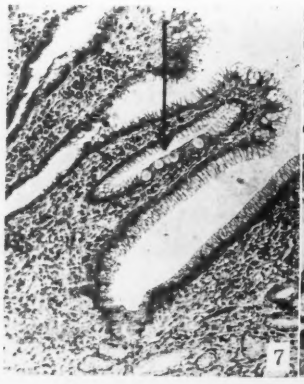
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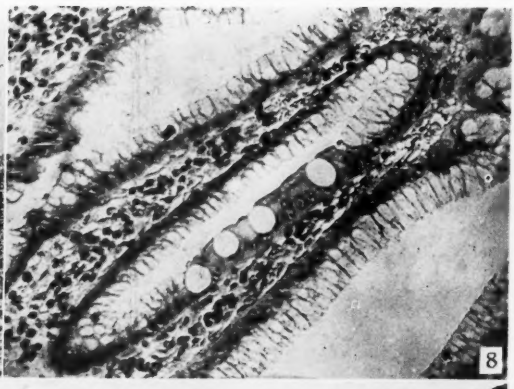
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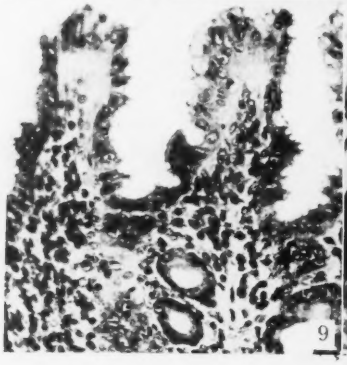
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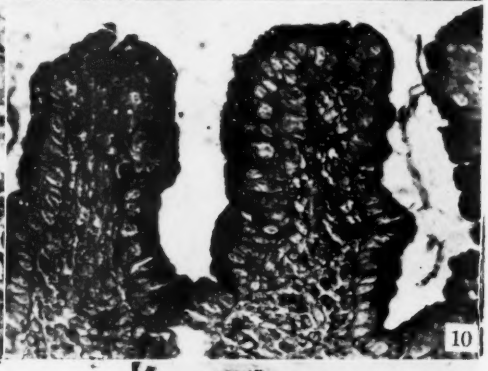
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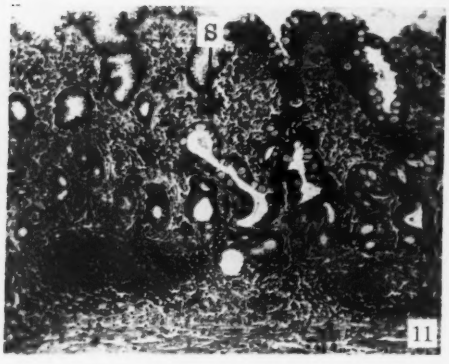
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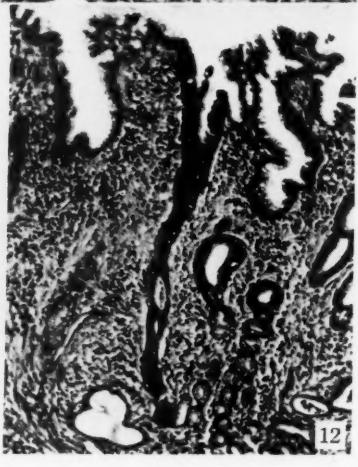
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[GANN, Vol. 46; March, 1955]

THE CYTOLOGICAL EFFECT OF CHEMICALS ON ASCITES SARCOMAS III. DAMAGE INDUCED BY PODOPHYLLOTOXIN, ALPHA-PELTATIN, BETA-PELTATIN AND QUERCETIN[1]

(With Plate IX)

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(Zoological Institute, Hokkaido University, Sapporo)

In one of the papers of this series, Makino and Tanaka (1953) described the inhibitory effects on the growth of rat ascites tumors by crude podophyllin, reporting that considerable damage to tumor cells with this drug and consequent temporary regression of tumor growth were induced with a prolongation of the life of tumor-bearing rats. This background naturally inspired interest in the possible effects of some crystalline compounds isolated from podophyllin on growth processes of ascites tumors. The compounds used in the present study are podophyllotoxin, alpha-peltatin, beta-peltatin and quercetin, which were kindly supplied through the courtesy of Dr. M. J. Shear, the National Cancer Institute, Bethesda, Md., U. S. A. They were isolated by Dr. I. L. Hartwell of the same institute from crude podophyllin. Previously, intensive damage induced in Sarcoma 37 in mice with these compounds was reported by Leiter, Downing, Hartwell and Shear (1950). Recently Makino and Cornman (1953) furnished evidence that different kinds of tumors showed different susceptibilities to podophyllotoxin.

This article constitutes one of the series of studies being conducted by Professor Sajiro Makino. Here the authors wish to acknowledge with cordial thanks the expert guidance and stimulating criticisms offered by Dr. Makino, and his kind personal aid in preparing this manuscript. The authors are also indebted to Dr. M. J. Shear of the National Cancer Institute, for his kindness in supplying the chemicals employed in this study.

It should be mentioned that the schedule of the following experiments was made by Tanaka and Kanô, together with the arrangement of the data obtained, according to the plan of Dr. Makino. The experiments were performed by Tonomura for podophyllotoxin, by Okada for alpha-peltatin, by Kanô for beta-peltatin, and by Umetani for quercetin.

Material and Methods

The effects of the four compounds, podophyllotoxin, alpha-peltatin, beta-peltatin

1) Contribution No. 319 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

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and quercetin, on the rat ascites tumors, MTK-sarcomas I, II and III,[1] were studied from the cytological viewpoint. The rats used for transmission of ascites tumor were pure bred Wistar albinos, weighing 80 to 120 gm. Intraperitoneal injections of aqueous solutions of chemicals were made in the tumor-bearing rats on the 4th to 6th day after the transplantation of the tumor. By that time the most active proliferation of the tumor cells had been attained in the peritoneal cavity of the animal. More than 70 tumor-bearing animals were employed in the experiments described in this paper.

For observations, smear preparations were made from a droplet of the tumor ascites obtained by abdominal puncture at appropriate intervals, beginning with 10 minutes and ending with 5 days, following the application of the chemical. At every time, sampling was made from both the untreated control tumor-rats and the treated ones. The slides were colored with acetic dahlia. Giemsa's preparations were also studied for comparison.

Mitotic Rates of Tumor Cells in Control Tumor-bearing Rats

The mitotic rates of tumor cells observed in untreated control tumor-bearing rats through the whole transfer generations are presented here for comparison with those examined in treated animals. Table 1 gives three examples of the mitotic rates for the MTK-sarcomas I, II and III, respectively.

Table 1.
Mitotic rates by day of tumor cells in control rats bearing the MTK-sarcomas I, II, and III.

Days after transplantation	Mitotic rates in percentage		
	MTK-I (%)	MTK-II (%)	MTK-III (%)
1	1.2	2.7	2.0
2	3.9	3.8	4.5
3	5.0	4.0	4.7
4	4.8	4.2	3.2
5	4.0	3.6	2.8
6	4.3	4.5	4.0
7	3.1	3.3	2.6
8	2.3	2.5	3.5
9	2.6	1.8	2.4
10	1.5	1.7	2.4
11	0.8	0.9	1.3
12		1.1	1.6

The average life days of the control tumor-bearing rats were 11.2, 12.0 and 10.5 for MTK-sarcomas I, II and III, respectively.

1) General characteristics of these ascites tumors were described respectively by Tanaka and Kanô (1951), Makino and Kanô (1953) and Umetani (1953).

Experimental Observations

1. Effect of podophyllotoxin: The aqueous solution of podophyllotoxin was prepared by dissolving the substance with a 50% aqueous solution of propylene glycol at about 50°C (Leiter et al. 1950). A single intraperitoneal injection was given to each tumor-bearing rat on the 6th day after transplantation at dose levels of 10 and 20 milligrams per 1000 grams of body weight.[1] Tumor samples were obtained at every 10 to 15 minutes during the first 3 hours, and then at every 2 or more hours after injection for a period of 4 to 5 days. The effect of the drug on the growth of the MTK-sarcoma II was observed cytologically.

Tumor-damaging activity of podophyllotoxin was found to be low at a dose of 10 mg/Kg. At a dose of 20 mg/Kg, the damage to tumor cells was induced in the tumor as early as 10 minutes after injection of the drug: an effect was observed in about 50 percent of the tumor cells observed. The tumor samples showed a number of tumor cells which blocked at metaphase. The early damage was characterized by abnormal condensation and irregular scattering of the metaphase chromosomes, agglutination of chromosomes, irregular processes or atypical amoeboid protrusions of the cytoplasm, blebbing of the cell surface, and the bridge-formation of sticky chromosomes at anaphase. These degenerative changes advanced with time. Thirty minutes after injection, almost all cells at metaphase showed chromosome clumping or further advanced degenerative features. By 45 to 60 minutes after injection, the disintegration of tumor cells was more marked; more than 70 percent of tumor cells under observation were damaged showing pycnotic aggregations of chromatin. By 90 minutes after injection, the damage was produced in about 80 percent of the tumor cells. By this time, the ascites was considerably reduced with consequent temporary regression of tumor growth. Figures 1 to 3 of Plate IX show the pictures of cell damage following the treatment with podophyllotoxin. Generally speaking, the damage to the tumor cells with the application of podophyllotoxin follows a similar pattern to that described for the effects of podophyllin by Makino and Tanaka (1953 a): generally the cell is blocked at metaphase and then undergoes pycnotic degeneration.

Four to five hours after injection, the cells in process of disintegration decreased in number. There were a certain number of tumor cells of small size in the ascites, which were characterized by a small amount of cytoplasm and a single, well-defined nucleus showing distinct nucleoli; probably they had persisted without being visibly damaged by the drug. By 7 hours, the small-

1) The dose levels are to be designated as 10 mg/Kg and 20 mg/Kg in the following descriptions.

sized, resistant tumor cells appeared in mitotic division. They divided in a regular mitotic manner. Morphological analysis of the chromosomes revealed that the chromosome complex of these cells was essentially similar to that found in the stem-cells of this tumor by Makino and Kanô (1953).

The number of dividing figures increased with time. By 15 to 18 hours after injection, many tumor cells appeared in active division. The increase in the number of tumor cells by division was followed by an accumulation of the tumor ascites. At about 24 hours after treatment, regrowth of the ascites tumor had been attained again in every treated rat with remarkable expansion of the abdomen. Table 2 illustrates the above account clearly.

Table 2.
The results of the experiment with podophyllotoxin at a dose level of 20 mg/Kg in the MTK-sarcoma II.

Time relation	Regular tumor cells		Damaged tumor cells (%)	Leucocytes in ascites (%)	Total number of observed cells	
	Mitotic cells (%)	Resting cells (%)			(No.)	(%)
Before injection	4.2	87.0	0	8.8	1161	100.0
After inject.						
10 min.	2.8	62.7	23.1	11.4	1241	100.0
20 min.	1.2	33.8	55.6	9.4	1100	100.0
30 min.	0.5	25.5	68.4	5.6	1057	100.0
45 min.	0.6	21.6	70.4	7.4	1049	100.0
60 min.	0	10.6	86.7	2.7	962	100.0
1.5 hrs.	0.1	12.2	83.5	4.2	1048	100.0
2.0 hrs.	0	5.2	92.8	2.0	985	100.0
3.0 hrs.	0	13.1	84.3	2.6	1073	100.0
5.0 hrs.	0	17.6	73.9	8.5	1094	100.0
7.0 hrs.	0.9	39.5	50.1	9.5	1140	100.0
10.0 hrs.	4.7	55.6	33.2	6.5	1094	100.0
12.0 hrs.	9.0	61.7	11.8	17.5	981	100.0
15.0 hrs.	14.2	52.2	0.1	33.5	1156	100.0
18.0 hrs.	16.6	58.2	0.3	24.9	1108	100.0
24.0 hrs.	2.3	80.7	0	17.0	990	100.0
48.0 hrs.	2.5	90.5	0	7.0	981	100.0

The tumors in untreated controls showed no induced damage.

From the evidence presented above, it can be stated that the cytological effects of podophyllotoxin are similar to those produced by podophyllin (Makino and Tanaka 1953 a), though the influence of the former drug does not last for a long time. Generally, within 24 hours following the application of podophyllotoxin, regrowth of the tumor occurs in the treated animals, no complete regres-

sion of the tumor having been observed. The life days of the treated tumor-bearing rats were from 16 to 18 days.

2. **Effect of alpha-peltatin:** The aqueous solution of alpha-peltatin was prepared by dissolving the substance first with 0.01 cc of N-NaOH and by diluting it with distilled water. On the 4th to 6th day after transplantation of the tumor, the tumor-bearing rat received a single intraperitoneal injection of the drug at dose levels of 20 and 25 milligrams per 1000 grams of body weight. Sampling of tumor was made in similar way as in podophyllotoxin-treatment. Both the MTK-sarcomas I and II were examined for evidence of damage in the following experiments.

At a 20 mg/Kg dose, tumor-damaging action of this drug was very weak. The following data were derived from experiments with a 25 mg/Kg dose.

The early damage to tumor cells was seen 10 minutes after injection of the drug. This drug exerted an injurious influence on cells both in the resting and in dividing stage. It induced abnormal agglutination of the chromosomes at metaphase and the breakdown of cytoplasm (Fig. 4). The cytoplasm showed

Table 3.
The results of the experiment with alpha-peltatin at a dose level of 25 mg/Kg in the MTK-sarcoma I.

Time relation	Regular tumor cells		Damaged tumor cells (%)	Leucocytes in ascites (%)	Total number of observed cells	
	Mitotic cells (%)	Resting cells (%)			(No.)	(%)
Before injection	2.9	97.1	0	0	2125	100.0
After inject.						
10 min.	3.4	26.1	52.5	17.9	963	100.0
20 min.	1.9	5.0	83.7	9.4	1063	100.0
30 min.	0.7	3.2	83.9	13.1	1044	100.0
45 min.	0.1	5.0	75.8	19.1	1035	100.0
60 min.	0.2	0.9	89.7	9.2	1096	100.0
1.5 hrs.	0	6.9	76.2	16.9	1019	100.0
2.0 hrs.	0	10.5	77.8	11.7	1008	100.0
3.0 hrs.	0	7.5	72.3	20.4	1038	100.0
5.0 hrs.	0	7.3	74.8	18.9	1068	100.0
7.0 hrs.	0.7	27.9	48.7	22.7	1119	100.0
10.0 hrs.	3.2	49.5	30.9	16.4	1095	100.0
12.0 hrs.	2.4	62.4	21.5	13.7	1019	100.0
15.0 hrs.	3.1	65.4	3.2	28.3	1119	100.0
18.0 hrs.	2.5	72.3	1.6	23.6	1086	100.0
24.0 hrs.	1.8	77.6	2.4	18.2	1064	100.0
48.0 hrs.	0.9	69.6	1.0	28.5	1123	100.0

severe distortion due to blebbing, and then its final breakdown resulted. With the passage of time, the clumping of the chromosomes into irregular masses leading to the death of the cell resulted.

The damage to the tumor increased with time with a similar pattern to that observed in the podophyllotoxin-treatment. By 7 to 10 hours after injection of the drug, there were large numbers of damaged tumor cells in the ascites, together with unaffected resistant tumor cells of small size. Within 10 hours afters injection, these resistant cells appeared in division (Fig. 5). Observations of the chromosomes confirmed that the chromosome complex of these resistant cells was essentially similar to that observed in the tumor-stem cells of MTK-sarcomas I and II. The dividing cells increased in number with time. By 15 to 20 hours after treatment, regrowth of the tumor had been attained again in every experimental animal. The data on which the above descriptions are based are summarized in Table 3.

From the above results it seems highly probable that the inhibitory effect on tumor growth by alpha-peltatin is less intensive than by podophyllotoxin, though the former drug has a higher order of toxicity to tumor cells than the latter. It is also noticeable that this chemical exerts its injurious influence upon both resting and mitoitc cells. With the application of this drug abnormal condensation, irregular scattering of chromosomes and pycnotic disintegration of nuclei occur along with the breakdown of cytoplasm.

In every experimental animal, no complete regression of the tumor has been attained. The life span of the treated animals ranged from 12 to 15 days.

3. Effect of beta-peltatin[1]**:** The aqueous solution of beta-peltatin was prepared similarly to that of alpha-peltatin, and a single intraperitoneal injection was made in each of the rats bearing both the MTK-sarcomas I and III at dose levels of 5, 10, 15, 30 and 50 milligrams per 1000 grams of body weight. Tumor samples were obtained after injection at intervals as indicated in Table 4.

It was found after preliminary trials that the tumor-damaging activity of this drug was low at the doses of 5 mg/Kg and 10 mg/Kg, and that the tumor-bearing rats could not survive with injection of the drug at dose levels of 30 mg/Kg and 50 mg/Kg. The following descriptions refer to the experiments carried out at the dose level of 15 mg/Kg.

Damage to tumor cells was observed as early as 10 minutes after injection of the drug. As was observed in the podophyllin-treatment, this drug first damaged the chromosomes at metaphase. Prophase cells showed no visible change at an early time. The division of almost all cells was blocked at metaphase, and the chromosomes were irregularly condensed and scattered in the cell without any

1) Thanks are due to Mr. Y. Ohnuki for his kind assistance in obtaining the data.

Table 4.
The results of the experiment with beta-peltatin at a dose level of 15 mg/Kg in the MTK-sarcoma III.

Time relation	Regular tumor cells		Damaged tumor cells (%)	Leucocytes in ascites (%)	Total number of observed cells	
	Mitotic cells (%)	Resting cells (%)			(No.)	(%)
Before injection	4.2	89.0	1.1	5.7	1000	100.0
After injection						
10 min.	4.3	61.7	19.4	14.6	1000	100.0
20 min.	1.7	46.7	37.9	13.7	1020	100.0
30 min.	1.0	45.8	40.0	12.5	1000	100.0
45 min.	0.4	40.3	53.9	5.4	1000	100.0
60 min.	0.9	44.3	45.7	9.1	1020	100.0
1.5 hrs.	0.7	47.5	45.3	6.5	1000	100.0
2.0 hrs.	0.3	52.2	39.1	8.4	1210	100.0
3.0 hrs.	0.7	44.7	43.7	10.9	1010	100.0
5.0 hrs.	0.5	30.5	59.9	9.1	1020	100.0
7.0 hrs.	0.3	21.4	68.7	9.6	1170	100.0
10.0 hrs.	0.4	27.9	65.6	6.1	1300	100.0
12.0 hrs.	0.2	21.5	70.0	8.3	1250	100.0
15.0 hrs.	0.2	10.5	82.2	7.1	1100	100.0
18.0 hrs.	0	7.7	83.5	8.8	1000	100.0
24.0 hrs.	0	5.7	59.2	36.1	1050	100.0
48.0 hrs.	0	6.2	4.2	89.6	1000	100.0
72.0 hrs.	0.3	2.8	0.4	96.5	1000	100.0

evidence of equatorial arrangement. By this time irregular processes of cytoplasm or blebbing of the cell surface were also observed in the resting cells. Such change continued during about 3 hours after injection of chemicals, in about 50 percent of the tumor cells observed. At a later time after injection, the chromosomes were observed to be shorter and more irregularly thickened, and at a still later stage they appeared as unusually deformed, bizarre bodies. At 5 to 7 hours following injection, the number of tumor cells which were arrested at metaphase showed a gradual increase, and at 12 to 18 hours their occurrence was most prominent (Figs. 6-7). Degenerative changes advanced with time: the condensed chromosomes were agglutinated into irregular masses being followed by pycnotic aggregation of chromatin, which induced death of the cell. Eighteen to 24 hours after injection, large numbers of damaged cells appeared in samples of the ascites tumor. With the accumulation of damaged cells the reduction of the ascites became remarkable. At 48 to 60 hours after injection, most of the debris from degenerating tumor cells had disappeared

from the ascites. By this time there appeared a number of leucocytes in the ascites, and only a few undamaged cells could be found mingled with them (Fig. 8). Thus, temporary regression of the tumor had been attained by 2 to 3 days after treatment with the drug, in striking contrast to the development of tumor in the untreated animals. Tumor samples of 3 days after injection contained a certain number of cells undergoing regular mitoses. By 3 to 4 days the regrowth of the tumor was attained. No complete destruction of tumor has been observed. Table 4 represents the data on which the above descriptions are based. In spite of a remarkable inhibition of the growth of the ascites tumor, the life span of the treated animals was considerably reduced, ranging from 7 to 10 days, due probably to the toxic effect of the drug.

From the results of the above observations, it can be said that the tumor-damaging activity of beta-peltatin tested for rat ascites tumors closely corresponds to that of podophyllin described by Makino and Tanaka (1953 a). The influence of the drug lasts for a long period of time, while the toxic effect of the drug is more striking compared with that of podophyllin.

4. Effect of quercetin: Similar to podophyllotoxin, the aqueous solution of quercetin was prepared by dissolving the substance with a 50% aqueous solution of propylene glycol (Leiter et al. 1950). A simple intraperitoneal injection was given to each rat bearing the MTK-sarcoma III on the 6th day day after transplantation of tumor, at dose levels of 20, 30, 35 and 40 milligrams per 1000 grams of body weight. Tumors were sampled every five to ten minutes after injection during the first 24 hours.

Preliminary examinations at the dose levels mentioned above revealed that the tumor-damaging activity of this drug was low at the doses of 20 mg/Kg and 30 mg/Kg, and that the tumor-rats could not survive at a dose level of 40 mg/Kg. The following data were derived mainly from the experiments made at a 35 mg/Kg dose.

Five to ten minutes after injection of the drug, the tumor samples showed a number of tumor cells arrested at metaphase. At a later time following injection, the chromosomes became thicker and shorter, and at a still later stage they appeared as unusually deformed bizarre bodies (Fig. 9). At 30 to 60 minutes after injection, almost all cells at metaphase exhibited chromosome agglutination. With the passage of time, degenerative changes of the tumor cells advanced. Three to 5 hours after treatment, large numbers of damaged cells appeared in samples of the tumor. By this time a temporary retardation of tumor growth seems to be attained as supposed from a decrease of the ascites. At 10 to 12 hours after injection, regrowth of the tumor had been caused by the proliferation of unaffected resistant tumor cells of small size (Fig. 10). By 15 hours after injection of the drug, regrowth of the tumor had been completed in every

Table 5.
The results of the experiment with quercetin at a dose level of 35 mg/Kg in the MTK-sarcoma III.

Time relation	Regular tumor cells		Damaged tumor cells (%)	Total number of observed cells	
	Mitotic cells (%)	Resting cells (%)		(No.)	(%)
Before injection	3.6	89.0	7.4	1067	100.0
After injecton					
10 min.	3.2	79.8	17.0	617	100.0
20 min.	2.4	86.1	11.5	612	100.0
30 min.	2.0	76.3	21.7	613	100.0
45 min.	1.8	86.3	11.9	633	100.0
60 min.	1.1	89.3	9.6	655	100.0
1.5 hrs.	1.2	79.7	19.1	610	100.0
2.0 hrs.	0.5	76.7	22.8	699	100.0
3.0 hrs.	0	65.4	34.6	656	100.0
5.0 hrs.	0	69.6	30.4	616	100.0
7.0 hrs.	0	72.9	27.1	648	100.0
10.0 hrs.	0.2	85.1	14.7	616	100.0
12.0 hrs.	0.5	85.9	13.6	647	100.0
15.0 hrs.	1.0	88.9	10.1	604	100.0
18.0 hrs.	1.3	87.4	11.3	605	100.0
24.0 hrs.	2.7	89.6	7.7	655	100.0
48.0 hrs.	1.7	80.3	18.0	605	100.0

treated rat. Table 5 indicates the data on which above account are based. The life span of the treated animals was from 14 to 16 days. No complete regression of the tumor was observed.

Based on the above results it is possible to say that the cytological effects of quercetin are generally similar to those produced by podophyllotoxin, though the tumor-damaging activity of quercetin is less intensive than podophyllotoxin, and also than alpha- and beta-peltatin.

Discussion and Conclusion

In the preceding paper of this series, Makino and Tanaka (1953 a) described the effects of podophyllin on cell division in the rat ascites tumors and its influence on the growth, and concluded that suppression of tumor growth and considerable prolongation of the tumor-bearing rat's life occurred as a result of damage to most of the tumor cells. However, some of the tumor cells remained unaffected and became the primary source of renewed malignant growth. A similar conclusion was attained by the same authors (Makino and Tanaka 1953 b) in a similar type of experiments with $CaCl_2$, $AlCl_3$ and H_2O_2, showing that there

occurred a temporary retardation of growth of the tumor in every experimental animal as a result of damage to the majority of the tumor cells, but a certain number of the resistant tumor cells always remained unaffected, and caused regrowth of the tumor by their multiplication. The major conclusion drawn from the present experiments with the application of four compounds of podophyllin, podophyllotoxin, alpha- and beta-peltatin and quercetin, is generally in agreement with that derived from the earlier studies by Makino and Tanaka (1953 a, b). By the application of every chemical examined for rat ascites tumors in this study, the temporary regression of tumor growth was brought about a greater or less degree in experimental animals due to damage to a large number of the tumor cells. But some of the tumor cells remained unaffected by the action of the chemicals. These persistent unaffected tumor cells constitute the primary source of the subsequent growth of malignant cells, leading to the reappearance of the tumor in the treated animals. No complete regression of growth of the tumor was observed.

The resistant tumor cells are generally characterized by a small amount of cytoplasm and a single well-defined compact nucleus with distinct nucleoli. They divide in an almost regular mitotic manner. Makino and Tanaka (1953 a, b) made detailed observations on the chromosomes of these tumor cells by morphological analysis of the individual chromosomes, and found that the chromosome complex of these cells was essentially similar to that observed in the stem-line cells of the tumor. In the present study, the chromosomes of the resistant tumor cells were investigated in the MTK-sarcomas I and II in the experiments with podophyllotoxin and alpha-peltatin. It was confirmed that the resistant tumor cells were none other than the stem-line cells of the tumor on the basis of the similarity of the chromosomes between the two types of cells.

It was found that the cytological effects of podophyllotoxin, beta-peltatin and quercetin were generally similar in response to those induced by podophyllin by Makino and Tanaka (1953 a). These chemicals first exerted influence on the division of the chromosome at the stage of metaphase; generally affected cells were blocked at metaphase, and then the pycnotic disintegration of nuclei with blebbing of the cytoplasm was induced. Alpha-peltatin was observed to affect both the chromosomes (or nuclei) and cytoplasm almost simultaneously. Makino and Tanaka (1953 a) have reported that a 0.1 percent solution of crude podophyllin with isotonic glucose solution injected into the peritoneal cavity of rats bearing ascites sarcoma acts as a mitotic poison, damaging the tumor cells at metaphase. Cell division is arrested at metaphase and chromosomes transform into irregular masses after condensation. Cornman and Cornman (1951) have studied the effects of podophyllin on cell division in some marine eggs, and have reported that podophyllin derivatives produce mitotic effects similar to those induced by

colchicine. Damage to tumor cells by the application of podophyllin and of its compounds as described above closely resembles that induced by the use of large doses of colchicine or by long exposures to the drug.

At the dose levels tested for rat ascites tumors in this study, podophyllotoxin, alpha-peltatin and beta-peltatin showed high tumor-damaging activity. Quercetin had a lower order both of activity and of toxicity. Leiter et al. (1950) in the investigation with mice bearing Sarcoma 37, and Cornman and Cornman (1951) in their work with marine eggs, obtained similar results. Makino and Cornman (1953) have shown that the type of response to malignant cells of podophyllotoxin and its severity vary with the type of tumor studied.

Among the podophyllin compounds tested in this investigation, beta-peltatin closely resembles podophyllin in the mode of action in the fact that the influence of these chemicals lasts for a long period of time.

Summary

This study deals with the effects of podophyllotoxin, alpha- and beta-peltatin and quercetin, all being crystalline compounds isolated from podophyllin, on cell division in the ascites tumors of rats and their influence on the growth of tumors. By the application of every chemical at appropriate dose levels, the temporary suppression of tumor growth was induced to a greater or less degree in experimental animals as a result of damage to a large number of the tumor cells. But some of the tumor cells remain unaffected by the action of chemicals in every case. These resistant unaffected tumor cells constitute the primary source of the subsequent growth of malignant cells leading to the reappearance of the tumor in the treated animals. No complete inhibition of growth of the tumor has been observed.

It was shown that the four compounds isolated from podophyllin were not identical in the order both of activity and of toxicity.

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Explanation of Plate IX

Photomicrographs of ascites tumor cells damaged by the chemicals. From smear preparation stained with acetic dahlia.

Figs. 1–3. Distortions of metaphase chromosomes of tumor cells in MTK-sarcoma II, induced by podophyllotoxin: 10, 20 and 30 minutes, respectively after injection of the drug. ×1100.

Figs. 4–5. Tumor cells of MTK-sarcoma I under treatment with alpha-peltatin. ×450. 4, showing breakdown of cytoplasm, 10 minutes after injection. 5, showing dividing figures of resistant tumor cells, 12 hours after injection.

Figs. 6–8. Tumor cells of MTK-sarcoma III following treatment with beta-peltatin. 6, advanced condensation of chromosomes in metaphase cells, 5 hours after injection. ×800. 7, disintegrating tumor cells, 18 hours after injection. ×500. 8, most of the damaged cells were absorbed, and one of the small-sized tumor cells (indicated by an arrow) was found unaffected among leucocytes, 48 hours after injection. ×350.

Figs. 9–10. Tumor cells of MTK-sarcoma III, after treatment with quercetin. ×500. 9, disintegrating tumor cells observed 1.5 hours after injection. 10, tumor cells taken 12 hours after injection, showing their proliferation.

要　旨

薬品の白鼠肉腫に及ぼす細胞学的影響，第 III 報

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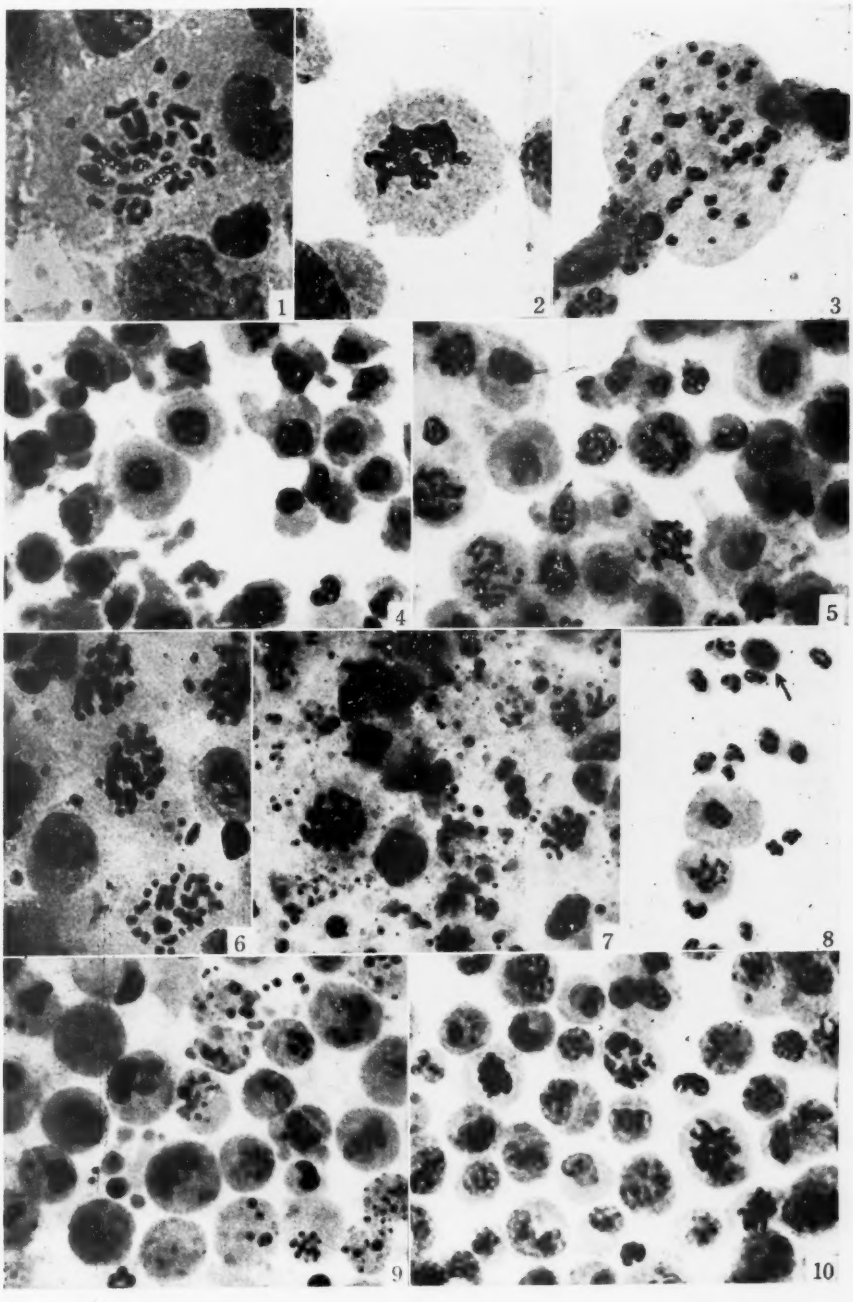
牧野・田中 (1953) はポドフィリンが MTK- 肉腫，ならびに吉田肉腫に対して顕著な制癌作用を有することを明かにした。われわれはポドフィリンより分離精製されたポドフィロトキシン，アルファ・ペルタチン，ベータ・ペルタテン，クエルセチンの 4 種の試薬について MTK-肉腫におよぼす影響を細胞学的に観察した。腫瘍移植 4～6 日目のラッテの腹腔内にそれぞれの薬品の適当量を注射すると，薬品の種類によって若干の程度の差異は認められるが，いずれの場合にも腫瘍細胞の多数に崩壊が起り，腫瘍の増殖は一時抑制される。その細胞学的作用はポドフィリンのそれに類似したもので，中期細胞の著しい分裂抑制がみられると同時に休止細胞に対する影響も観察された。しかしながら，薬品の影響をうけることなく生きのびる一群の腫瘍細胞が存在していて，それらが再び増殖して腫瘍再成の源となる。実験においては，肉腫の成長の完全な退行は一例も観察されなかったが，僅かながら生存期間の延長をもたらした。すなわち，腫瘍細胞におよぼす影響はいずれの薬品もポドフィリンにはおよばないが，4 種の中ではポドフィロトキシンが最も強烈であり，ついでベータ・ペルタチン，アルファ・ペルタチン，クエルセチンの順に作用の度合が減少した。

(文部省科学研究費による)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[GANN, Vol. 46; March, 1955]

SOME OBSERVATIONS ON A NON-TRANSPLANTABLE ASCITES TUMOR DEVELOPING IN AN INBRED MOUSE* **
(With Plate X)

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Ascites tumors of rats and mice have been becoming a useful tool for the cytological investigations, a considerable number of papers having been published by Yosida 1949 a, b, 1951, 1952 a, b, 1954 a, b, c, d, Makino 1951 a, b, 1952 a, b, Makino & Yosida 1949, 1951, Makino & Kanô 1951, 1953, Tanaka & Kanô 1951, 1853, Hauschka 1953, Levan & Hauschka 1952, 1953, Hauschka & Levan 1953, Klein 1951, Klein & Klein 1951 and Bayreuther 1951. Some of the ascites tumor were induced by application of azo dyes, such as the Yoshida sarcoma, MTK-sarcoma I, II and III, while some others developed from spontaneous solid tumors of unknown origin by transforming into a fluid form as occurred in Takeda sarcoma, Hirosaki sarcoma and some mouse ascites tumors.

While working with the breeding experiments of mice, in the Makino Laboratory, the present authors found a fluid tumor of spontaneous origin which developed in the peritoneal cavity of one of the inbred mice derived from the B-strain. Repeated attempts have been made to transmit this fluid tumor using mice of either original B-strain or other strain, but there has been no success transplanting it in any mice used. The present paper is to report the results of transplantation with notes on the histological and cytological diagnosis of this fluid tumor.

Here it is the authors' pleasant duty to acknowledge their indebtedness to Professor Sajiro Makino for his kind guidance and revision of the manuscript.

Material and Method

The B-strain of mice in which the tumor developed is of a hybrid origin; its ancestors consisted of a pair of hybrid mice between common albino mouse (*Mus musculus*) and wild house mouse (*Mus molossinus*). This strain was supplied through the courtesy of Dr. Kondo, Tokyo University. They have been inbred by sister-brother cross in the Makino Laboratory since 1946 (Yosida 1952 b).

The implantation of this fluid tumor was made following the method usually employed for the ascites of rats, injecting with a sharply pointed glass pipette

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a small amount of tumor ascites into the peritoneal cavity of the host. For the transplantation of the tumor tissue, the ordinary transplantation needle was used.

Observations

1. *Record of the tumor-bearing mouse:* On the 7th of October 1951, the authors' attention was called to a mouse of the B-strain which showed an unexpected growth of its abdominal cavity. By puncturing the abdominal cavity with a fine pipette it was revealed that the abdominal expansion was due to an accumulation of hemorrhagic ascites. Microscopical observations indicated that the ascites contained many tumorous cells. Several attempts were made to transmit the tumor cells by the injection of tumor ascites in mice of the original B-strain, as well as those of some other strains. But all mice which received the injection failed to produce the tumor as described below. On the 9th, this animal showed a diseased condition, so that it was sacrified for autopsy. It was found that considerable hypertrophic changes had taken place in spleen, liver, lymphatic gland and kidney with unusual dark brown coloration. A tumorous mass was developed in the dorsal side of the peritoneal cavity.

2. *Results of transplantation:* The tumorous cells inoculated in the peritoneal cavities of new hosts underwent degeneration soon after transplantation, without showing any sign of multiplication. The tumor pulps which were prepared from the tumorous mass and implanted in the epidermis of hosts also showed degeneration without exception. Results of transplantations are summarized in Table 1.

Table 1. Results of transplantations of the tumor

Material	Strain of host		Positive transplantation
	B	Other strains	
Ascites tumor	10*	10	0
Pieces from tumor mass	5	5	0
Liver Pieces	5	5	0
Kidney pieces	5	5	0
Spleen pieces	5	5	0

* Number of mice which received the transplantation.

3. *Histological observations of the visceral organs of the tumor-bearing mouse:* Some histological observations were made on the visceral organs of the tumor-bearing mouse.

Liver: Lymphoid cells, lymphocytes and neutrophil leucocytes were found

infiltrated into the Glisson's sheath (Fig. 1 in Plate XI). They were very few in the hepatic lobes. Around the central veins there were many lymphoid cells. Liver cells were more or less atrophilic in appearance, while the Kupffer star-cells had remained unaltered in their morphological feature.

Spleen: Remarkable infiltration of lymphoid cells was observable.

Kidney: In the Malpighian bodies of the kidney there were many lymphoid cells. They infiltrated especially into and around the Bowman's capsules (Fig. 2).

Tumorous mass: The tumorous mass found in the dorsal side of the peritoneal cavity was made up mainly of cells which looked like lymphoid cells. They showed many mitotic figures. In addition to them there were some histiocytes and neutrophil leucocytes (Fig. 3).

Based on the histological observations, it is assummed that this fluid tumor may be a sort of lymphatic leukemia, though no definite statement can be made.

4. *Cytological observations of the ascites tumor:* The fluid tumor was studied with smear preparations stained with acetic orcein. The tumor ascites contained many tumorous cells with an appearance similar to the lymphoid cells. The nuclei were compact and round in appearance (Figs. 4–7). Mononucleate cells were of common occurrence together with a few bi- or tri-nucleate cells. Many mitotic figures were found in these cells; the chromosomes of these tumorous cells showed many abnormalities of which stickiness and coalescence of chromosomes, deformation of chromosomes into unusual bodies, and displacement and abnormal orientation of chromosomes at metaphase were striking. There were no cells with regular metaphase configuration.

Remarks

As indicated in the above descriptions, the fluid tumor here concerned is non-transplantable, many attempts having failed to transplant it in any mice so far examined. There are some possible interpretations for the cause of non-transplantability of this tumor: 1) the strain of the mouse used for transmission may not be completely pure, 2) the tumorous cells are highly differentiated, and 3) this tumor contains no cell undergoing regular mitosis. It seems to the authors that the last assumption may be most probable for the explanation of this non-transplantability. A series of karyological observations on several tumors carried out by Makino and his co-workers (Makino 1951, a b, 1952 a, b, Makino and Kanô 1953, Makino and Tanaka 1953, etc.) and by one of the present authors (Yoshida 1953, 1954 a, b, c, d), have developed the concept of stem-line cells as progenitors of the neoplastic population. Each tumor was characterized by a stem-cell lineage (or lineages) having characteristic chromosome pattern. The growth of the tumor in transplantation is primarily due to the multiplication of the stem-line cells. Probably, non-transplantability of the

present fluid tumor may be primarily due to the absence of stem-line cells with characteristic chromosome pattern.

Summary

A fluid tumor, probably a sort of lymphatic leukemia developed spontaneously in a mouse of the B-strain. The mouse showed an expansion of the body cavity resulting from an accumulation of ascites containing large numbers of lymphoid cells. Some visceral organs such as the liver, kidney, and spleen showed a remarkable infiltration with tumorous cells; there was also a tumorous mass found in the dorsal side of the peritoneal cavity. After several trials it was found that this fluid tumor was non-transplantable. The non-transplantability of this tumor may probably be due to the absence of the stem-line cells which contribute to the growth of the tumor in transplantation.

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Explanation of Plate

Photomicrographs of the tumor tissue.

Fig. 1. Section of liver, showing lymphoid cells, lymphocytes and neutrophil leucocytes infiltrated in the Glisson's sheath.

Fig. 2. Section of kidney, showing many lymphoid cells infiltrated into and arround the Bowman's capsules.

Fig. 3. Section of tumorous mass found in the peritoneal cavity, showing many lymphoid-like cells.

Fig. 4. 4-7. Tumor cells in resting and in process of division.

要　旨

マウスに発生した非移植性腹水腫瘍について

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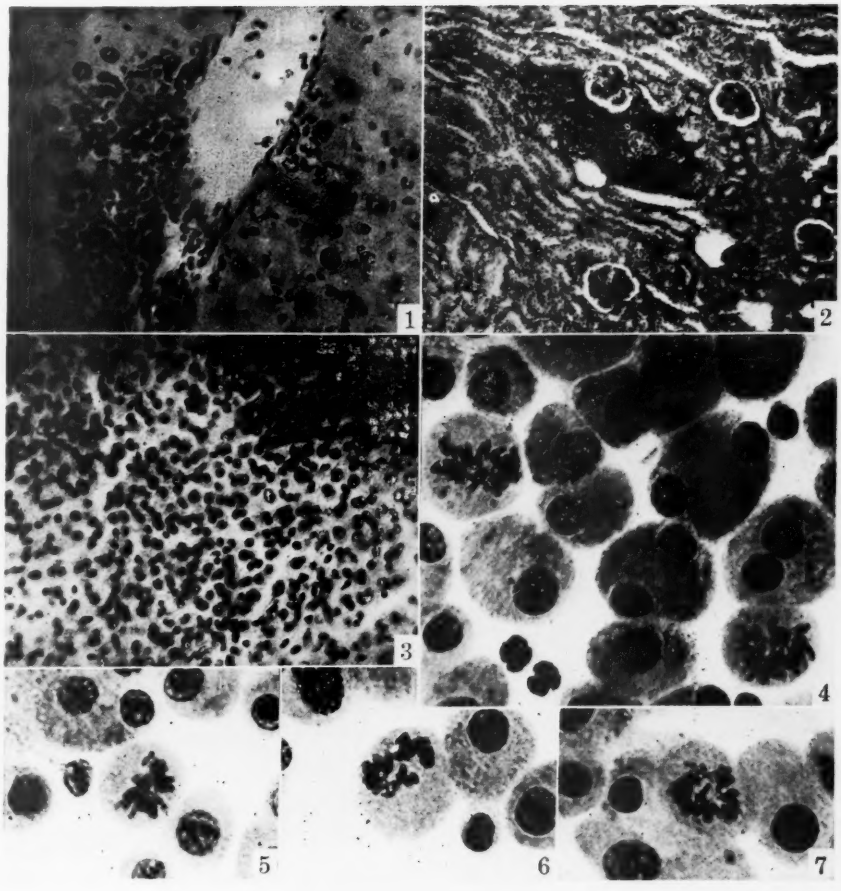
筆者の一人，吉田が北大牧野研究室でB系マウスの近親交配をしていたときに，その系統の一個体に腹水性腫瘍の自然発生を見た。この腫瘍を原発系統および他系統の多数の個体へ移植を試みたが，結果はすべて陰性であった。この研究はこの腫瘍の組織細胞学的な検索の結果である。

脾臓，肝臓および腸間膜淋巴腺などに著しい肥大が認められ，腹腔背壁には拇指頭大の癌状腫瘍が観察された。腹腔内には多量の出血性腹水が認められた。肝臓，脾臓，および腎臓の組織学的観察を行ったが淋巴性細胞，淋巴球および中性好性白血球の浸潤が見られた。特に腎臓の変化が強度で，腎小体に淋巴性細胞の浸潤が著しい。腹腔背壁の癌状腫瘍は淋巴性細胞および淋巴球からなり，分裂細胞が多数見られた。

腹水を aceto-orcein で固定染色し観察したところ，多数の腫瘍細胞が見られ，分裂像もしばしば観察された。分裂細胞の染色体の形態を観察したが，凝集型，その他の異常分裂型が多く，正常に分裂している所の，いわゆる分裂型細胞は一個体も見られなかった。

以上の観察結果から，この腫瘍は恐らく淋巴性白血病であろうと考えられる。この腫瘍が他の個体に移植されなかった理由としては色々と考えられるが，正常に分裂増殖するところの，分裂型細胞の欠除がその一因ではなかろうかと考えられた。　(文部省科学研究費による)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[GANN, Vol. 46; March, 1955]

DESAMINASE ACTION ON FATTY ACID AMIDES IN LIVERS OF RATS FED WITH HEPATIC CARCINOGENS

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Since three years our laboratory has been engaged in the study of desaminase activity in the liver of rats fed on hepatic carcinogens. In these series of works halogenated fatty acid amides have been used as substrates. We now studied the desaminase activity by using simple fatty acid amides instead of halogenated derivatives, as it was readily expected that the rat-liver extract may also decompose the simple fatty acid amides. This idea was strengthened by the work of Bray et al. They have confirmed already, that the rabbit-liver extracts and slices have the desaminase activity on following amides: n-butyramide, n-valeramide and phenylacetamide; whereas formamide, acetamide and propionamide were hydrolysed to a very small extent.

Materials and Methods

Experimental animals.——DAB rats. Albino rats of Wistar strain and of mixed strain of our laboratory stock were used for the experiments. Rats with initial weight of about 100 g were placed on the diet containing 0.06 per cent of 4-dimethylaminoazobenzene (DAB) for about 150 days. After that, the DAB feeding was interrupted and the rats replaced on normal diet for 30–40 days.

AAF rats. The rats, maintained on diet containing 0.05 per cent 2-acetylaminofluorene (AAF) for 150–180 days, were fed on normal diet for additional 90–120 days. At that time survivors of both carcinogen fed rats were sacrificed for experimental use.

Normal rats. Untreated rats of similar age which have been placed on normal diet (rice grains) throughout the time of experiment, were taken as control. Normal and carcinogen fed rats were allowed to take routine food and water *ad libitum* and occasionally supplemented with dried fish and green vegetables.

Substrates.——As substrate 13 aliphatic amides and 2 cyclic amides were employed in this study. They were prepared in our laboratory according to the usual synthetic method and were given in Table 1 with their melting points. Acetamide, benzamide and lauramide (m. p. 100°C) were omitted from the table owing to their unsuitable properties, i. e., the former two substances were hardly disrupted by the rat-liver extract and the latter one underwent non-enzymatic hydrolysis while the measurment is being carrying out.

Hardly soluble or almost insoluble substrates in water were dissolved in propylene glycol to get their solutions.

Enzyme preparation.——The hepatic tissues used for enzyme studies were removed from the animals immediately after decapitation and blotted dry, weighed and homogenized in Potter tubes. Then the homogenates were diluted with distilled water 5, 10 or 20 times of original fresh tissue according to the property of the substrates mixed.

The constituents of digestion mixtures and the conditions of incubation were given in Table 1. The mixtures were prepared in large pyrex test tubes with rubber stopper. After the incubation at 38°C for 4 or 24 hours ammonia evolved from the substrates was determined by Folin's method. The blank tests were made solely with liver extract, substituting the substrate solutions by the same amount of water or propylene glycol. The blank figures were subtracted from the above experimental data.

Table 1.

Homologous fatty acid amides and cyclic amides	m.p. (C)	Amount of substrate		Enzyme preparation		Phosphate buffer solution 0.1M (pH 9)	Time in hrs. for incubation at 38°C
		Mol.	cc	Dilution	cc	cc	
1) Propionamide	77.5°	1/10	5	5×	4	3	24
2) n-Butyramide	114°-116°	1/10	5	5×	4	3	4
3) Isobutyramide	126°	1/10	5	5×	4	3	4
4) n-Valeramide	106°	1/10	5	5×	2	3	4
5) Isovaleramide	138°	1/10	5	5×	4	3	24
6) n-Caproamide	101.5°	1/10	5	10×	2	3	4
7) Isocaproamide	119°	1/10	5	10×	2	3	4
8) Pelargonamide*	92°	1/10	2	20×	2	3	4
9) Caprylamide*	105°-107°	1/10	2	20×	2	3	4
10) Capramide*	99°	1/10	2	20×	2	3	4
11) Myristamide*	103°-105°	1/10	2	5×	2	3	24
12) Palmitamide*	107°	1/10	2	5×	2	3	24
13) Stearamide*	107°	1/10	2	5×	2	3	24
14) Phenylacetamide	155°-157°	1/50	10	5×	4	3	24
15) Phenylpropionamide	102°	1/50	10	5×	2	3	4

Constituents of digestion mixtures and conditions of incubation are represented. Substrates noted by stars, 8) to 13), are dissolved in propylene glycol instead of distilled water, because of their very scant water solubility. In these cases the same amount of propylene glycol without the substrates were used as blank tests.

Chart 1 Substrate: Propionamide (1)
Incubation: 24 hrs.

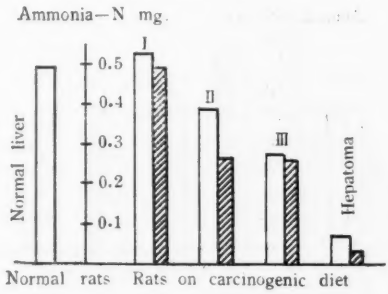


Chart 2 Substrate: *n*—Butyramide (2)
Incubation: 4 hrs.

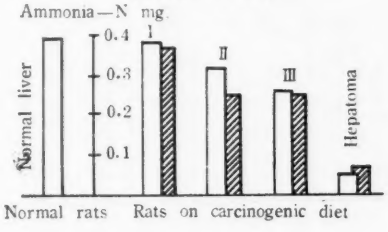


Chart 3 Substrate: Isobutyramide (3)
Iucubation: 4 hrs.

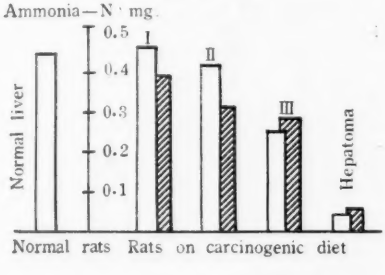


Chart 4 Substrate: *n*—Valeramide (4)
Incubation: 4 hrs.

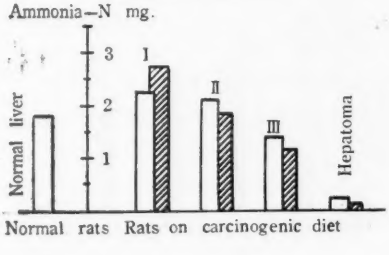


Chart 5 Substrate: Isovaleramide (5)
Incubation: 24 hrs.

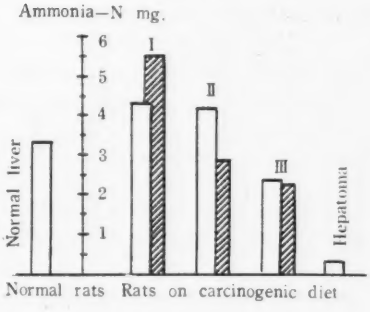


Chart 6 Substrate: *n*—Caproamide (6)
Incubation: 4 hrs.

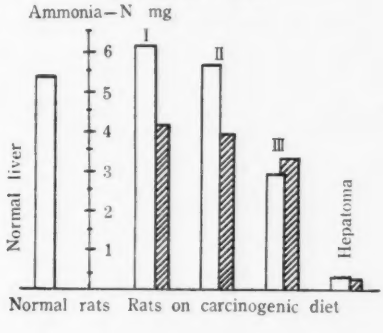


Chart 7 Substrate: Isocaproamide (7)
Incubation: 4 hrs.

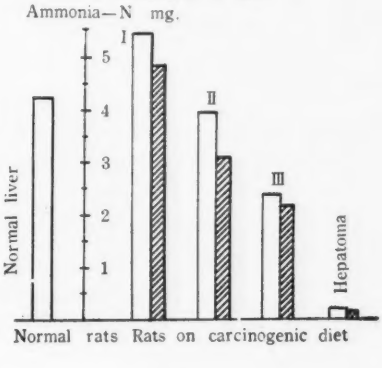


Chart 8 Substrate: Pelargonamide (8)
Incubation: 4 hrs.

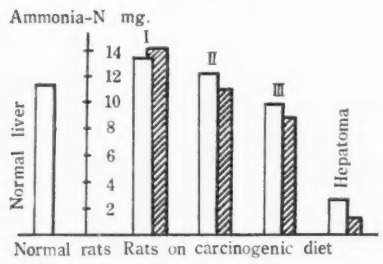


Chart 9 Substrate: Caprylamide (9)
Incubation: 4 hrs.

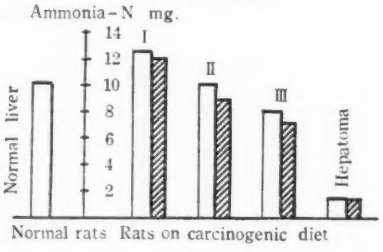


Chart 10 Substrate: Capramide (10)
Incubation: 4 hrs.

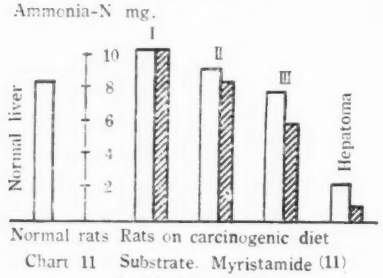


Chart 11 Substrate. Myristamide (11)
Incubation: 24 hrs.

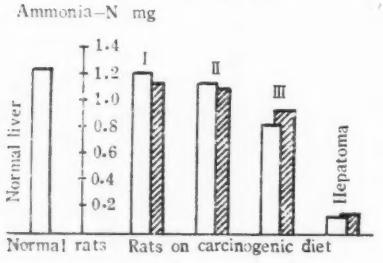


Chart 12 Substrate: Palmitamide (12)
Incubation: 24 hrs.

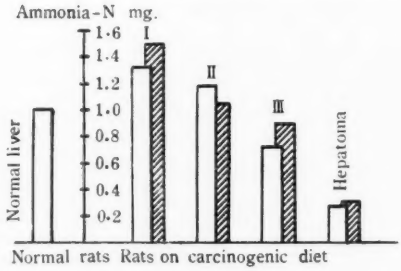


Chart 13 Substrate: Stearamide (13)
Incubation: 24 hrs.

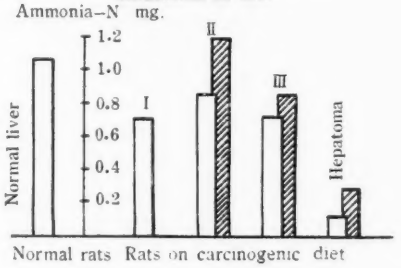


Chart 14 Substrate. Phenylacetamide (14)
Incubation 24 hrs.

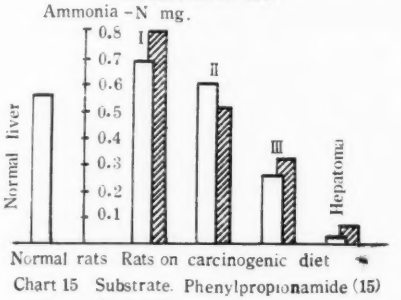
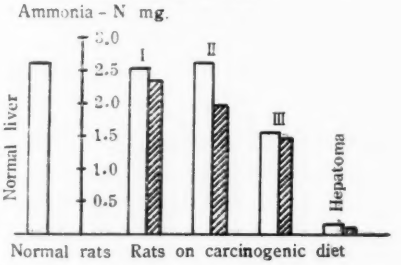


Chart 15 Substrate. Phenylpropionamide (15)
Incubation: 4 hrs.



Explanation for Charts 1—15

Desaminase activities of pathological hepatic tissues for 13 aliphatic fatty acid amides and 2 cyclic amides are represented in Charts 1-15 respectively, being compared with that of normal liver.

Ordinate represents the evolved ammonia-N mg per g of fresh hepatic tissues at pH 9 after 4 or 24 hrs. incubation at 38°C and the unit of graduation in ordinate is taken arbitrary for each Chart according to the activity.

Unshaded bars in the right side of ordinate represent the activities of hepatic tissues of DAB rats and shaded bars represent that of hepatic tissues of AAF rats.

The pathological but non-cancerous livers are classified according to the grade of their lesions into three groups, namely, I, II and III in both carcinogen fed rats. For further details see the text.

The bars under hepatoma represent the activity of hepatoma induced by DAB (unshaded) and by AAF (shaded) respectively.

Results and Discussion

The pH-activity curves of homologous fatty acid amides using normal liver extract were presented nearly as "bell-shaped", and the peak was situated about at pH 9 (Charts A and B). In consequence the following activities have been measured solely at pH 9 throughout.

Chart A

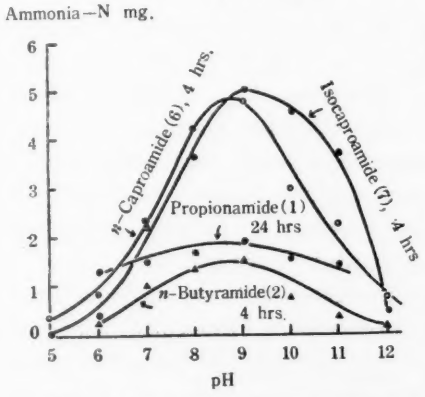
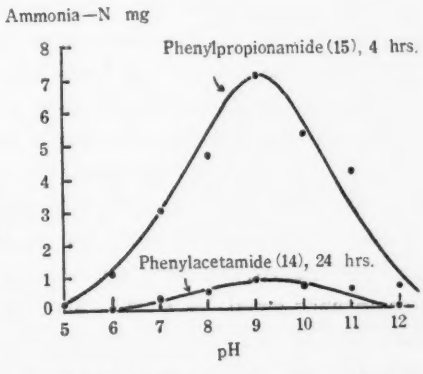


Chart B



pH-Activity curves of some readily water soluble aliphatic amides (Chart A) and 2 cyclic amides (Chart B) are represented. Ordinate shows the liberated ammonia-N mg per g of fresh normal liver, after 4 or 24 hrs. incubation at 38°C. In every curve the peak is observed about at pH 9.

Mean values of experiments were shown graphically in Charts 1 to 15, where the activities of normal liver, mean values of 10 to 11 individuals were represented, and in case of pathological but non-cancerous livers the activities were calcurated from the mean values from 5 to 6 individuals, and that of hepatoma obtaided from 1 to 3 rats.

In this connection the following explanations may be necessary. Liver lesions were classified grossly into two groups, namely, pathological but non-cancerous liver and hepatoma, as above mentioned. For the sake of facilities in representing in Charts 1 to 15, pathological but non-cancerous livers were subclassified according to the grade of lesions in three further groups, in both carcinogen fed rats, namely, grades I, II and III. (I) Macroscopically normal liver, (II) liver with uneven surface, (III) cirrhotic liver in DAB rats. In case of livers of AAF rats, they were classified according the idea of Wilson et al. as follows, (I) liver of no hyperplasia, (II) liver of nodular hyperplasia, (III) liver of marked nodular hyperplasia.

Normal liver homogenate digested 13 aliphatic amides and 2 cyclic amides markedly, although its enzymatic activity varied to some extent according to the substrates tested. Concerning water soluble straight chained aliphatic amides, the more rich in carbon atoms the more easily they were decomposed by normal liver extract as follows: n-caproamide (6) > n-valeramide (4) > n-butyramide (2) > propionamide (1).

Cipfers in the parentheses are the same number of substrates in Table 1 and those of in Charts. They are also used in the following lines.

In cyclic amides we obtained similar results: phenylpropionamide (15) > phenylacetamide (14).

In case of substrates very slightly soluble or almost insoluble in water, on the contrary, the results were quite different: stearamide (13), palmitamide (12), myristamide (11) < capramide (10), caprylamide (9) < pelargonamide (8). This order appears to be based upon on the molecular configuration of substrates. The digestability was inversly proportional to their increasing molecular weight. But in this case the substrates were slightly soluble even in propylene glycol, especially in compounds with higher molecule; accordingly the digestion conditions might be inadequate for the comparison of the activities.

The difference of the enzymatic activity between straight chained and branched compounds could be seen in the experiments using caproamides (6), (7) and valeramides (4), (5) except butyramides (2), (3); i. e., straight chained compound was more readily decomposed by normal liver extract than branched; the results were quite similar to the observations in the halogenated fatty acid amides as we previously reported.

While the lesions of the liver remained limited in extent, it showed some times higher activity than normal level, unrelated to the sorts of carcinogens added to the feed. When the lesion of liver developed to cirrhotic stage, the activity usually decreased, but still it maintained the appreciable activity. Hepatoma nodulus induced either by DAB or AAF, however, showed always very slight activity and was 1/4 to 1/30 that of normal lebel, though it fluctuat-

ed according to the sorts of substrates used. These aspects were closely related to the results obtained by their halogenated derivatives in our former experiments.

Summary

1) The enzymatic desamination of 13 homologous fatty acid amides and 2 ω-phenyl fatty acid amides in the liver extract of rats on hepatic carcinogens, 4-dimethylaminoazobenzene or 2-acetylaminofluorene, has been investigated.

2) Aqueous homogenate of normal liver had the marked activity to evolve ammonia from the above mentioned substrates, although the amount differed according to the sorts of substrates employed.

3) The pathological but non-cancerous liver showed the appropriate activity even in the case of marked alteration, as long as the neoplastic transformations had not taken place.

4) Hepatoma had very low activity, scarcely reaching about 1/4 to 1/30 that of normal liver according to the sorts of substrate employed.

5) So far as the straight chained fatty acid amides which could be used as aqueous solutions are concerned, the more easily they were cleaved by liver extracts the richer they are in carbon atoms, and the straight chained compounds were decomposed more readily than the branched with corresponding molecular weight.

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要　旨

肝癌生成物質投与ダイコクネズミ肝の脂肪酸アミド脱アミノ酵素について

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発癌過程における臓器の病変とその酵素活性度との相関関係の研究である，われわれはさきに発癌剤投与ネズミ肝の酵素アミダーゼに属するハロゲン化脂肪酸アミド脱アミノ酵素の活性度について報告した。ここに基質として単なる脂肪酸アミドの同族列 15 種類を選んだ。うち 2 種は ω-phenyl 脂肪酸アミドである。

発癌剤を長期間投与した後ネズミを正常食にもどして飼育をつづけた動物の肝を材料とした。肝の均質液と緩衝液と基質の水溶液――水に難溶性の物質は propylene glycol を溶媒に用いた――を混合し一定時間内に脱アミノ化されて遊離したアンモニアを Folin 法で定量し酵素の活性度とした。これはすべて白紙試験値を差引いてある。

対照とした正常動物肝の活性度は高い，病変肝（肝癌を除く）は病変の亢進に伴って漸時低下するがなおかなり高い活性度をもつ．肝癌そのものは活性度は極はめて低くく正常値の 10 分の 1 の程度である。この結果は既報ハロゲン化脂肪酸アミドの脱アミノ酵素と近似している。なお基質脂肪酸アミドの分子量，分子構造と酵素的分解性にも論及した。

(厚生省厚生科学研究費による)

[GANN, Vol. 46; March, 1955]

CHOLINE OXIDASE ACTIVITY IN THE HEPATIC TISSUES OF RATS FED ON CARCINOGENS

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(Director: Prof. Sanji Kishi)

Many works have been carried out, in the field of biochemistry aiming to elucidate the choline metabolism. The theme has been focused, in recent years, on the biochemistry of tumors, and noteworthy articles were publicated by many workers. Among them Woodward has investigated choline oxidase activity of livers of rats fed on carcinogenic azo dye, 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB).

The present author investigated this problem using pathological livers including hepatoma of rats, which had been fed carcinogenic diet. The normal appearing livers which were obtained from the rats in their early stages of experimental feeding on carcinogen were also taken up.

METHODS

Experimental animals. Carcinogen fed rats. White rats of Wistar strain and some mixed strain of the laboratory stock were placed for a long period of time on the diet incorporated with 0.06 per cent 4-dimethylaminoazobenzene (DAB) or 0.05 per cent 2-acetylaminofluorene (AAF). Duration of feeding of DAB rats was 120 days and that of AAF rats was 150–180 days. At the end of that time the rats were removed from carcinogenic diet and were placed on basal one without carcinogens for some additional days, i.e., 50–60 days for DAB rats and 90–120 days for AAF rats.

Rats fed on both carcinogenic diets in their early experimental days (1–5 weeks) were also employed.

Normal rats. Untreated rats which have been kept on basal diet were used.

All rats were allowed to take food and water *ad libitum* and occasionally supplemented with fish meal and green vegetables.

Enzyme preparation. The animals were killed by decapitation and exsanguination, after which the hepatic tissues were removed and weighed and homogenized by refrigeration. To homogenates thus obtained cool distilled water was added to obtain exact 5 times dilution of each original fresh tissues. The diluted homogenates were filtrated through cotton wadding and used at once as enzyme preparation without any further procedure, such as centrifugation or dialysis.

An aliquot part of the preparation was dried and weighed for the calculation of enzyme activity against dried weight.

Determination of choline oxidase activity. Oxygen uptake was measured at 38°C under pure oxygen atmosphere in Warburg manometric apparatus. In the main compartment of the flask were pipetted 1 cc of 0.1 M phosphate buffer of pH 7.5 and 3 cc of 0.025 M choline chloride solution (corresponding to 10.5 mg), and in the side arm was pipetted 1 cc of aquous homogenate of tissue and tipped in after 15 minutes equilibration.

The rate was corrected for the oxygen uptake of a similar mixture in the absence of choline chloride.

Activity was calculated from the initial linear portion of the rate curve and expressed as oxygen uptake c mm per hour per mg of dried tissue homogenate (Qo_2). The activity of tissue homogenate should be better calculated, based upon dried tissue, because the water content of pathological, especially cancerous, tissue is far higher than that of normal liver.

The measurment was carried out at 15', 30' and 45' after the process has been started and the value at 30' was multiplied by 2 to obtain that of one hour.

Results

Results were summarized in Tables 1 and 2, and the mean values were represented graphically in Charts 1 and 2. The description which stand under them may suffice for the explanations.

Discussion

Woodward has studied already the choline oxidase activity in the livers of rats during carcinogenesis by the feeding of 3'-Me-DAB, an extremely effective azo dye.

In this paper the results were discussed, performing the similar experiments by using azo dye, DAB, and a compound of the different category, AAF. Moreover the author payed especial attention to the way of feeding of rats. Rats were kept on carcinogenic diet long enough to induce neoplastic transformation of livers, then the carcinogen feeding was stopped, and the change of livers was observed after this succeeding prolonged feeding on normal diet.

Choline oxidase activity of pathological but non-cancerous livers showed comparable height as that of normal liver and so far as the lesion of liver developed to cirrhosis the activity was still high although there were some signs of diminution. These findings were inconsistent to those of Woodward. It may be due to the fact that she used the livers taken while the rats were being fed on carcinogen, 3'-Me-DAB. When the neoplastic transformation has taken place the activity falls markedly, to nearly 1/10 of normal lebel, regardless of whether

it has been induced by DAB or by AAF. This finding was nearly akin to the observations of Woodward and some other investigators such as Viollier, Kensler and Langemann.

Cholin oxidase activity of livers of rats in their early DAB feeding period decreased every week and reached the lowest point at the 3rd or 4th week of feeding, reaching 1/3 of the normal lebel. In case of AAF rats, after the first week of feeding, the activity was rather high ih comparison to that of normal liver, and moreover quite similar to that of DAB rats just described above. These findings are in close agreement with Woodward's, because her 3'-Me-DAB rats showed marked retardation of the activity after only 14 days feeding and maintained almost the same activity during further prolonged feeding.

Summary

1) Activity of choline oxidase in the hepatic tissues of rats fed carcinogens, 4-dimethylaminoazobenzene (DAB) and 2-acetylaminofluorene (AAF) was investigated.

2) The choline oxidase activity of the abnormal but non-neoplastic hepatic tissues showed similar height as that of normal liver, and when the grade of lesions developed to cirrhosis still maintained the moderate lebel although there occurred some indication of lowering. On the contrary as soon as hepatic tissues have once turned neoplastic they showed the extremely low activity, which was less than about 1/10 of normal lebel.

3) The choline oxidase activities decreased every week immediately after the experiment had been started, and reached the bottom at the 3rd or 4th week of feeding and were about 1/3 of the normal lebel.

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Table 1

Choline oxidase activity of livers of rats fed on carcinogenic diet for a long period of time, being compared with that of normal liver.

Normal rats	Carcinogen fed rats				
				DAB	AAF
4.14±0.645 (11)	Liver findings	Pathological but non-cancerous livers	I	3.77±0.419 (5)	4.27±0.325 (2)
			II	2.49±0.764 (6)	4.17±0.502 (6)
			III	2.98±0.329 (10)	2.88±1.417 (6)
		Hepatoma		0.73±0.404 (4)	0.46±0.318 (4)

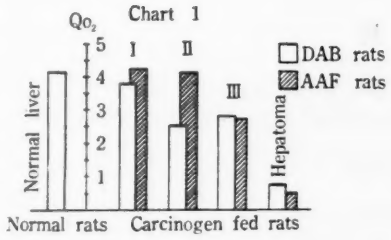
Table 2

Choline oxidase activity of livers of rats on carcinogenic diet in their early experimental period.

Normal rats	Carcinogens fed rats		
	Feeding days in week	DAB	AAF
4.14±0.645 (11)	1	3.47±0.911 (4)	4.70±0.644 (4)
	2	2.31±0.729 (5)	2.34±0.558 (4)
	3	1.43±0.697 (5)	2.15±0.766 (5)
	4	1.73±0.527 (5)	1.66±0.840 (6)
	5	1.49±0.297 (4)	1.80±0.624 (4)

Explanation for Tables 1 and 2

Cipfers represent O_2 uptake in c mm/hr/mg dried tissue homogenate. Mean values with standard deviations and number of animals (in parentheses) for corresponding animals are shown in Tables 1 and 2.



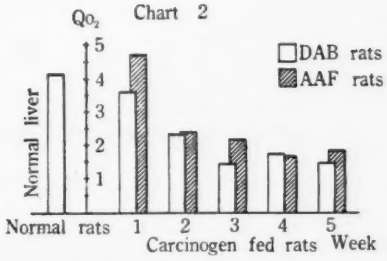


Chart 1

Choline oxidase activity at pH 7.5 of hepatic tissues of the rats fed with carcinogens, DAB or AAF, in comparison with that of normal liver, is graphically reprented.

Ordinate shows the rate of O_2 uptake (c mm/hr/mg dried tissue homogenate).

PatLological but non-cancerous livers are classified according to their grade into three groups, (I) macroscopically normal liver, (II) liver with uneven surface, (III) cirrhotic liver in DAB rats. Livers of AAF rats are classified according to the idea of Wilson et al. as follows, (I) liver of no hyperplasia, (II) liver of nodular hyperplasia, (III) liver of marked nodular hyperplasia.

The activities of pathological but non-cancerous liver (I, II and III) show nearly the same height as that of normal liver as long as the neoplastic transformation does not take place.

Chart 2

Choline oxidase activity at pH 7.5 in the livers of rats on carcinogenic diet (DAB or AAF diet) in the early experimental period. Ordinate shows the rate of O_2 uptake (c mm/hr/mg dried tissue homogenate).

Retardation in activity is shown every week until the 3rd or 4th from the time of starting carcinogen feeding.

要　旨

肝癌生成過程にをけるダイコクネズミの肝コリンオキシダーゼについて

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発癌剤を長期間投与し投与を中絶してから正常食に戻してなお長期間飼育をつづけたダイコクネズミの肝を材料とした。また一方投与実験初期でしかも投与継続中のダイコクネズミ肝を材料とした。

肝性組織均質液，コリン塩酸塩水溶液 (基質)，燐酸緩衝液 (pH 7.5) の混合液を 38° C にをいて検圧計で測定し Oo_2 でコリンオキシダーゼ活性度を表示した。

病変肝で発癌にいたらないものは活性度は正常値とさして遜色ない程度である，そして硬変肝ではやや低下の傾向がある。肝癌自体の活性度は顕著に減少している。なお発癌剤投与開始 3–4 週でしかも投与継続中のネズミ肝——肉眼的には病変は認められない——では著明に低下している。これらの結果から Woodward (1951) の報告を考えてみると彼の使用した動物の肝は常に発癌剤の作用下にあったことを思わせる。発癌剤自体の投与中絶えず実験動物の肝に惹起しつつあると考えられる毒作用と，その結果として現われた肝の病変とを区別して考えるべきである。

(文部省科学研究費による)

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STUDIES ON THE ENZYMATIC SYNTHESIS OF p-AMINOHIPPURIC ACID BY THE LIVER OF RATS FED 2-ACETYLAMINOFLUORENE

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The synthesis of hippuric acid from benzoic acid and glycine has been demonstrated in the slice of kidney and liver of guinea pigs, rabbits, rats and dogs by Borsook and Dubnoff (1). Recently, Cohen and McGilvery showed that a synthesis of *p*-aminohippuric acid from *p*-aminobenzoic acid and glycine by the cell-free homogenates of the liver of rats is possible (2, 3). It would appear to be of interest to determine the activity of this enzyme in the liver of rats during the course of the hepatic carcinogenesis because it seems that the synthesis of hippuric acid by cell-free preparations represents a convenient model for the study of the synthesis of the peptide bond in the liver cells.

EXPERIMENTS

A group of normal male rats of Wistar strain, weighing 120-150 g, was maintained on rice diet containing 2-acetylaminofluorene at a level of 0.06 per cent. The 2-acetylaminofluorene was dissolved in 30 *ml* of polyethylene glycol, the solution mixed evenly into the ingredient of the diet, consisting of 960 g of polished rice, 30 g of fish powder, 5 g of sodium chloride and 5 g of cod liver oil.

After 5 months on the experimental diet, animals were placed on the normal rice diet without the carcinogen and were reared for additional 2 months on it. As a control group, Wistar rats were fed on the same diet without the carcinogen. In the both groups, water was freely and green vegetables were occasionally supplied.

Animals were exsanguinated by cardiac puncture under the anesthetization by chloroform. All livers were removed as quickly as possible, and placed on ice and inspected. The livers were classified grossly according to the description of Wilson and associates (4) into four grades, slight, moderate, and marked hyperplasia and liver cancer as in the previous papers (5, 6, 7). If the liver contained both cancerous areas and relatively normal tissue, the different portions were dissected and handled separately.

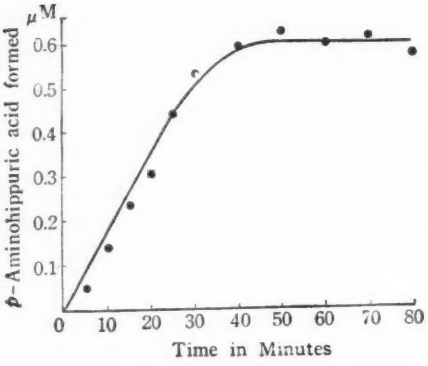
500 *mg* of the liver tissue weighed precisely by a torsion balance as quickly as possible. Homogenization of the liver tissue with 2 *ml* of cold isotonic

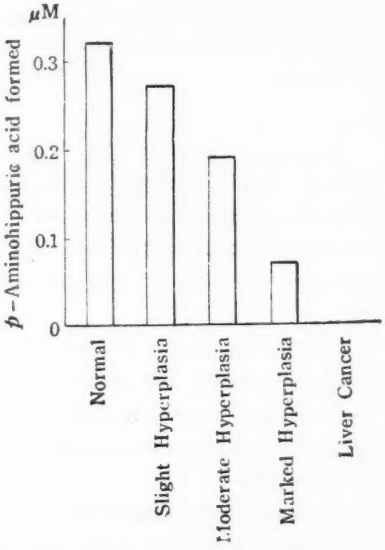
potassium chloride solution, buffered at pH 7.5 by 0.05 M potassium phosphate as the suspending medium, was carried out in ice-cold water by a glass homogenizer, and it was continued at least for 2 minutes after the tissue had been broken up. Usually one *ml* of this homogenate was used as the enzyme solution.

The *p*-aminobenzoic acid dissolved in hot alcohol and recrystallized from an alcohol-water mixture was used. Fumaric acid was recrystallized from the boiling diluted hydrochloric acid. Other reagents used were commercial products.

p-Aminobenzoic acid was dissolved in 0.1 M potassium phosphate buffer solution (pH 7.5), and fumaric acid and glycine were dissolved in distilled water, and then neutralized with potassium hydroxide.

The reaction mixture consists of 0.001 M *p*-aminobenzoic acid, 0.015 M glycine, 0.0007 M magnesium chloride, 0.05 M potassium phosphate and 0.0025 M fumaric acid. The media were made isotonic with potassium chloride into a final volume of 4.0 *ml* per flask.





p-Aminohippuric acid and *p*-aminobenzoic acid were determined by the method of Cohen and McGilvery (2), namely, the differential extraction of *p*-aminobenzoic acid from *p*-aminohippuric acid was made with ether at pH 3.9 and the colorimetric analysis was followed by a modification of the method of Bratton and Marshall (8).

The liver homogenates were added to the cold reaction mixture in a flask which was kept on ice until the incubation was started. The incubation was

carried out at 37°C for 25 minutes. When the incubation terminated, 4 *ml* of 0.6 N trichloroacetic acid was added to the mixture and then filtered. An aliquot volume of the filtrate was used for analysis of *p*-aminohippuric acid and *p*-aminobenzoic acid.

In this experiment, the reaction system was slightly different from that of Cohen. Both adenylic acid and cytochrome c were not added to the mixture and the gas phase was air during the period of incubation. As is shown in Fig. 1, the reaction was not continued more than 40 minutes from the beginning in spite of adding fumarate. Then it seemed most suitable to incubate for 25 minutes to determine the activity of the enzyme in this procedure.

The results of the experiment were summerized in Table 1, and were plotted in Fig. 2. It may be clearly seen that the activity is a little lower than the normal at the experimental stage, when the liver shows hyperplasia of slight degree, and moderate hyperplastic liver shows only half the activity of the normal. However, in the liver of marked hyperplasia or annular cirrhosis, the enzyme was decreased to one-fifth in the activity. Moreover, when the tissue turned into cancerous, no synthesis of hippuric acid was demonstrated.

Discussion and Conclusions

The present study is an attempt to ascertain the relative activity of the enzymatic synthesis of *p*-aminohippuric acid in the course of the production of liver cancer by feeding of 2-acetylaminofluorene.

Table 1. Activities of the enzyme of *p*-aminohippuric acid synthesis. (Figures given as averages and ranges)

Liver Findings	No. of Experiment	*p*-Aminohippuric acid formed (μM)	Activity*
Normal	13	0.32 (0.15–0.43)	100
Slight Hyperplasia	12	0.27 (0.12–0.38)	84
Moderate Hyperplasia	9	0.19 (0.10–0.30)	59
Marked Hyperplasia	12	0.07 (0.02–0.12)	22
Liver Cancer	6	0.00 (0.00–0.00)	0

* Values for normal taken as 100.

It was demonstrated that the faculty of the hippuric acid synthesis of the liver homogenates declined rapidly according pathological changes of the liver of rats fed 2-acetylaminofluorene and no activity was observed when the tissue has turned into cancerous as described above. The result might suggest that this synthetic enzyme is localized only in the active liver cells. Then, it is reasonable that the activity of synthetic enzyme shows a considerable reduction in the liver of an extensive hyperplasia in which active liver cells may be reduced in number owing to the proliferation of the connective tissue and the bilary cysts filled with fluid when compared to the normal. No investigation of the enzymatic synthesis of hippuric acid in the course of the production of the liver cancer has hitherto been reported.

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要　旨

肝癌生成過程における白鼠肝 *p*-Aminohippuric acid 合成酵素について

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2-Acetylaminofluorene を白鼠 (ウィスター系) に与えて，肝癌を生成する過程における肝の *p*-Aminohippuric acid 合成酵素の活性度をしらべた。

実験結果を総括的にいえば酵素活性度は肝の病変に応じて極めて鋭敏な態度を示した。すなわち肝の病変の進行につれて階段的に減少し肝癌組織では全くみられなかった。

この酵素はいわゆるペプチッド結合 (CO-NH) を合成するのであるが肝の癌性化につれてその活性度が減弱して行くことは興味あることと思われる。

(厚生省科学研究費による)

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CATALASE DEPRESSING TISSUE FACTORS: TOXOHORMONE AND KOCHSAFT FACTOR

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INTRODUCTION

In 1948, Nakahara and Fukuoka (1, 2) succeeded in extracting a liver catalase depressing factor, the so-called toxohormone, from malignant tumor tissues. The substance has not yet been obtained in chemically pure state, but it is thermostable, non-heat coagulable, water soluble and alcohol insoluble, and its concentrate in the form of amorphous powder markedly lowers the liver catalase activity when injected into normal mice. Fukuoka and Nakahara (3) later published the observation that the effect of the injections of toxohormone can be counteracted to some extent by liver powder feeding or administration of iron, and suggested that toxohormone may interfere with the utilization of iron which is needed for the synthesis of catalase. This hypothesis, which attributes the mode of action of toxohormone to the inhibition of catalase synthesis, was extended by these authors to the cases of other iron-containing enzymes and hemoglobin, and they pointed out the possibility that the inhibition of these synthetic processes may be the true cause of cancer cachexia.

A different mode of action was suggested in 1952 by Hargreaves and Deutsch (4), who reported that tumor *kochsafts* directly inhibit the catalase activity *in vitro*, using crystalline catalase. In 1953, Hirsch and Pfützer (5), using ascites tumors, confirmed the existence of toxohormone by *in vivo* experiments and the direct catalase inhibition by *kochsaft* by *in vitro* experiments. More recently, Price and Greenfield (6) measured the amount of liver catalase in tumor bearing animals by isolating the catalase, and demonstrated the actual decrease. This finding supports the theory of the inhibition of catalase synthesis.

We considered it important to clear up the relation between toxohormone of Nakahara and Fukuoka and the so-called *kochsaft* factor of Hargreaves and Deutsch, and our experiments showed that the factor in the tumor *kochsaft* which inhibits crystalline catalase *in vitro* is by no means specific to tumor tissues but is easily demonstrable also in such normal tissues as rat liver, spleen, kidney and embryo. We also obtained a toxohormone concentrate, according to the method of Nakahara and Fukuoka, which was active in 10 mg doses *in vivo*

and found it to be almost entirely without effect *in vitro*. Conclusion was reached, therefore, that toxohormone and *kochsaft* factor are different entities.

Material

Tissue extracts (*Kochsaft*), toxohormone concentrate and crystalline catalase used in the present experiments were prepared as follows:—

Tissue extracts (*Kochsaft*): For normal tissues, spleen, liver, kidney, testis, embryo, placenta, and blood plasma from normal rats were used, and malignant tumor tissues used were transplanatable hepatoma (rat), Umeda rhodamine sarcoma (rat), Yoshida sarcoma (rat), NF sarcoma (mouse) and Ehrlich ascites carcinoma (mouse). Twice the volume of water was added to the tissue, and homogenized by means of a Potter-Elvehjem type homogenizer. Homogenate was gently boild over water bath for 15 minutes after which absolutely or almost absolutely clear solution was separated by centrifugation, first at 3000 r. p. m. for 5 minutes and then by means of high speed centrifuge at 10,000 g for 15 minutes.

Toxohormone: The usual toxohormone fraction (alcohol precipitate) obtained from rhodamine sarcoma was washed in cold with N/10 HCl, and again with alcohol and dried. This sample was found to be active for normal mice in 10 mg doses (Chart 1).

Crystalline catalsase: This was prepared from beef liver according to the method of Shirakawa (7).

As to other materials used in a part of the experiments, glutathione (GSH) was a preparation of the Kirin Beer Laboratory, almost 100% reduced form according to amperometric examination. Egg albumin was that of Takeda Pure Chemicals, Ltd. (Tokyo).

Experimental Methods

In testing the effect of *kochsaft* and other substances on the activity of crystalline catalase we followed closely the original procedure of Hargreaves and Deutsh. After allowing the enzyme solution to react with tumor *kochsaft* at 0°C for 2 hours, the catalase activity of the reaction mixture was determined by the method of Euler and Josephson (8), and the reaction velosity constant K was obtained. For a part of the experiments, Shirakawa's "dropping method" (9) was used.

Experimental Results

Experiments were repeatedly many times, always with consistent results, and typical examples of each kind of experiments are given in Tables 1, 2 and 3. In these tables, the results of experiments are expressed in the term of the

percent decrease of catalase activity, taking the figure of blank control as 100.

Table 1. The Inhibition of Crystalline Catalase by Normal Tissue *Kochsaft*.

Tissues	Concentration of *kochsaft* in reacting medium % (v/v)	Percent decrease of activity
Liver (1)	5	34.1
	10	53.2
	20	61.2
Liver (2)	10	48.1
Spleen (1)	5	21.3
	10	32.3
	20	53.8
Spleen (2)	20	83.4
Kidney	20	60.5
Testis	20	57.3
Embryo	10	57.0
	20	74.5
Placenta	20	71.9
Blood Plasma	10	38.5
	20	62.1

Table 2. The Inhibition of Crystalline Catalase by Tumor Tissue *Kochsaft*.

Tumors	Concentration of *kochsaft* in reacting medium % (v/v)	Percent decrease of activity
Transplantable	5	25.2
Hepatoma (rat)	10	33.0
	20	36.0
Umeda Rhodamine	5	30.5
Sarcoma (rat)	10	61.5
	20	85.8
Yoshida	5	89.5
Sarcoma (rat)	10	92.1
NF-Sarcoma (1)	5	36.8
(mouse)	10	85.3
	20	96.9
(2)	10	76.9
	20	91.5
Ehrlich Ascites		
Fluid (Raw state)	20	27.8
(Boiled)	20	73.6

Table 3. The Inhibition of Crystalline Catalase by Toxohormone, GSH and Egg Albumin.

Substances	Concentration in reacting medium	Percent decrease of activity
Toxohormone	2.5 mg/ml	0.8
	13.4 mg/ml §	9.0
Glutathione	13.2 mg/dl §§	25.3
Egg Albumin (Raw state)	200 mg/dl	0.0
(Boiled)		51.9

§ This concentration was considered as sufficient, as the preparation used was active *in vivo* in 10 mg dose per mouse.

§§ This amount corresponds to the concentration of GSH in *kochsaft* when prepared at 1 : 2 ratio, assuming the GSH content of liver to be 200 mg/100 g.

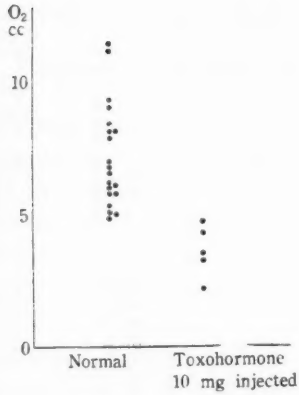

Chart 1. Potency of toxohormone prepared from Umeda rhodamine sarcoma. The measurement of catalase in this experiment was carried out as Nakahara and Fukuoka (1).

Table 4. The Inhibition of Crystalline Catalase by Tissue *Kochsaft* in Relation to GSH Contents.

GSH concentration	Percent decrease of activity
$0.3\times10-3$ M	3
$1\times10-3$	10
$3\times10-3$	22
$10\times10-3$	44

Tissues	Percent decrease of activity	GSH concentration in *kochsaft* (M)	Percent decrease of activity expected from the GSH concentration in *kochsaft*
Testis	17.6	1.5×10^{-3}	79.6
Kidney	35.7	1.1×10^{-3}	32.2
Liver	29.1	1.86×10^{-3}	56.7
Spleen	28.0	0.67×10^{-3}	14.3
Embryo	23.0	0.7×10^{-3}	33.4

In all cases, GSH solution and tissue *kochsaft* were added as much as 20% (v/v) in catalase solution, and left at 0°C for 2 hours. Then catalase activity was assayed by the dropping method of Shirakawa (9). The GSH content of tissue *kochsaft* was determined by amperometric titration method as described by Goldzieher (10).

As may be clear from Tables 1 and 2, the *in vitro* inhibition of catalase is observed not only by *kochsaft* of malignant tumors but also by that of normal tissues, and there seems to be no special different in the degree of inhibition as to the two different sources. *Kochsaft* prepared from normal blood plasma was found to be about as effective as that similarly prepared from the cell-free fluid of Ehrlich ascites carcinoma. Moreover, as shown in Table 3, egg albumin solution, normally inactive, yieled actively inhibiting supernatant after it was boiled and centrifuged. These results raise questions as to the interpretation

of Hirsch and Pfützer's *in vitro* experiments.

Our experiments concerning the role of glutathione, which is expected to be contained in *kochsaft*, proved some inhibition when incubated with enzyme solution in amounts corresponding to the expected concentration in the *kochsaft* calculated from the assumed concentration of 200 mg/100 g in the liver tissue. We also measured amperometrically the amounts of GSH in *kochsaft* prepared from various normal rat tissues, and compared the results with the catalase inhibiting action of corresponding *kochsaft* (Table 4). It was found that GSH effect can account for the inhibition only partially; while almost 80% of inhibition by testis *kochsaft* can be explained by GSH, *kochsaft* from spleen and embryo with high inhibiting action showed rather low GSH content.

Discussion

The clear-cut results of the above experiments hardly require any comment. Certainly the existence of Hargreaves and Deutsch's tumor *kochsaft* factor, which inhibits *in vitro* the activity of crystalline catalase is adequately confirmed, but at the same time it was demonstrated that *kochsaft* of normal tissues, such as liver, spleen, kidney, embryo and placenta, are just as potent as tumor *kochsaft* in inhibiting the catalase activity *in vitro*. These results definitely prove that Hargreaves and Deutsch's *kochsaft* factor is non-specific, being common to all tissues, malignant as well as normal.

In this connection, it may be recalled that tissue extracts in general contain glutathione and other substances capable of reducing $Fe^{\cdots}$ of catalase to $Fe^{\cdot\cdot}$, and that such substances may well play a part in the catalase inhibition *in vitro*. Indeed, our experiments showed that at least a portion of the inhibiting action of liver *kochsaft*, for instance, may be explained as being due to its glutathione content.

Another point of interest in our experiments is in the demonstration of the inhibiting action of *kochsaft* prepared from normal blood plasma or egg albumin solution. This fact would cast doubt on the interpretation of Hirsch and Pfützer's results with the ascites fluid of Ehrlich carcinoma, since it seems unnecessary to assume the presence of a tumor specific factor in the ascites fluid to account for the results. Further studies are needed to satisfactorily understand these phenomena.

The lack of catalase inhibiting action *in vitro* on the part of toxohormone concentrate is in agreement with the early results of Nakahara and Fukuoka (2), and its inability to inhibit the activity of crystalline catalase *in vitro* must be regarded as sharply differentiating it from the *kochsaft* factor.

The mechanism of catalase depression *in vivo* by toxohormone is imperfectly understood. Price and Greenfield (6) showed that the depression of liver catalase

in tumor bearing animals is due to the actually smaller amount of catalase present, rather than to the inhibition of the activity. Fukuoka and Nakahara (3) suggested that toxohormone may in some way interfere with the synthesis of catalase. Although the possibility cannot be absolutely excluded that toxohormone may in final analaysis be found to act directly upon catalase *in vivo*, there is at present no information to support such a possibility.

Summary and Conclusion

In confirmation of Hargreaves and Deutsch, we found that tumor *kochsaft* factor clearly inhibits *in vitro* the activity of the solution of crystalline catalase, but it was also demonstrated that *kochsaft* prepared from normal liver, spleen, kidney, embryo, etc., brings about similar degree of inhibition. These facts, taken together with the inability of a potent toxohormone fraction to produce *in vitro* inhibition of crystalline catalase solution, seem conclusive in showing that the so-called *kochsaft* factor is an entirely different entity from toxohormone.

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要　旨

カタラーゼ抑制性組織因子：トキソホルモンと Kochsaft 因子

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中原，福岡により悪性腫瘍から分離されたトキソホルモンは，福岡，中原の肝粉食または鉄投与により，その作用が打消されるという実験結果から，カタラーゼの合成阻害を起すものと推定され，一般にトキソホルモンにより含鉄酵素，ヘモグロビンの合成阻害が起り，それが癌悪液質の原因になると考えられる。これに対して，Hargreaves and Deutsch は悪性腫瘍の Kochsaft が in vitro で結晶カタラーゼを直接阻害すると報告した。トキソホルモンと Kochsaft 因子との相関関係を明らかにすることは重要だが，今回，我々の実験結果から，Kochsaft 因子は正常組織にも悪性腫瘍と同程度に存在し，その一部はグルタチオンによるものであり，腫瘍特異的でなく，さらにトキソホルモンは in vitro で結晶カタラーゼを阻害せず，両者は全く異ったものであることが確認された。これは Price and Greenfield が担癌動物の肝臓から分離したカタラーゼ量が実際少いという実験結果と共に合成阻害説を支持するものである。

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ON THE ANTI-CANCER ACTION OF QUINONE DERIVATIVES

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One of us (Fukuoka[1]) has been conducting screening tests for possible chemotherapeutic agents for cancer by a simple and practical technique for determining the relative cancericidal action *in vitro*, and we examined the anti-cancer effect of various quinone derivatives by means of the same technique.

Certain quinones are known to be carcinogenic, though very weakly (Takizawa[2]), while, according to the postulate of Haddow[3], it is possible to expect cancer inhibiting effect from carcinogenic substances. Although the true cause of this paradox has not been explained, and no strict parallelism exists between carcinogenic and carcinostatic actions of given substances, we considered it worth while to investigate a rather wide range of quinone derivatives for their anti-cancer actions. Previous experiments in this field include those of Badger[4] et al., with 1, 4-naphthoquinone, 2-methyl-1, 4-naphthoquinone, and sodium-1, 2-naphthoquinone sulfonate, of Powell[5] with 1, 2-dihydroxy-1, 4-naphthoquinone, anthraquinone and 1, 2, 4-trihydroxyanthraquinone, of Lewis and Goland[6] with 1, 4-diaminoanthraquinone and 1, 4, 5, 8-tetra-amino-anthraquinone and, finally, of Haddow and Robinson[7] with 1, 2, 4, 5, 6, 8-hexahydro-anthraquinone. These authors, excepting Badger, reported a certain small degree of tumor inhibiting effect. Von Euler and Hasselquist[8] also noted tumor inhibition after small doses of quinones.

The compounds used in the present study were mostly the derivatives of 1, 4-naphthoquinone, all prepared in our laboratory. With few exceptions, their anti-cancer actions have not hitherto been tested, so far as we know. We first found, by *in vitro* technique, that alkylthio derivatives of 1, 4-naphthoquinone have definite cancer cell killing action, and then tested the therapeutic effect *in vivo* of these potent compounds, using Ehrlicha scites carcinoma, according to the conventional method.

Experimental Method

Method of *In Vitro* Test. 10 to 14 day old well growing subcutaneous grafts of transplantable NF mouse sarcoma were used. Substance was tested at the concentrations of 0.05 %, 0.01 %, and 0.005 % in physiological salt solution, adjusting the reaction to pH 6.0-7.0 by means of sodium bicarbonate. As control,

physiological salt solution of pH 7.0 was used. Approximately the same amount of NF sarcoma tissue, cut up into small fragments of about 1 mm diameter, was immersed in these various mixtures in small Petri dishes, and was left to stand at 4-7°C for 24 hours. At the end of this period, the sarcoma tissue fragments from the four dishes (with three different dilutions and control) were implanted by means of trocar into the subcutaneous tissue at four different sites of one mouse.

At least three mice were used for a single test compound, and the growth of tumors resulting from the implantation was observed for two weeks. If control graft did not take, the case was discarded as being a naturally immune mouse.

Method of *In Vivo* Test. Freshly aspirated ascites fluid of Ehrlich mouse carcinoma, one week after the inoculation, was injected in 0.05 cc doses intraperitoneally into groups of usually five normal mice each of about the same body weight, and the treatment was started 48 hours after the ascites injection. The treatment consisted of daily injections in physiological salt solution of 10-20 mg per kg of the substance to be tested. When the substance was water insoluble, the suspension was stabilized by adding sufficient amount of finely pulverized carboxymethyl cellulose to the salt solution.

Table 1. Effect of Thio-quinone Derivatives on the survival *in vitro* of NF mouse sarcoma.

Compounds			Tumoricidal effect at		
No.		M. p.	0.05%	0.01%	0.005%
1885	Methylthio-quinone	140~145	3/3	—	—
1889	2,3-Dimethylthio-quinone	210~212	3/3	—	—
1883	2-Methylthio-1,4-naphthoquinone	175~178	6/6	6/6	5/6
1929	2-methylthio-1,4-naphthoquinone diacetate	144	3/3	3/3	1/3
1886	2,3-Dimethylthio-1,4-naphthoquinone	143	6/6	6/6	3/6
476	2-Ethylthio 1,4-naphthoquinone		3/3	3/3	1/3
1902	2-Carboxymethylthio-1,4-naphthoquinone	180~182	3/3	3/3	—
1933	2-Carboxymethylthio-1,4-diacetoxynaphthalene	150~155	2/2	—	—
1998	2-Methyl-3-carboxymethylthio-1.4-naphthoquinone	148~149	3/3	—	—
1920	2-Thioacethylenglycol-1,4-naphthoquinone	125~127	3/3	3/3	1/3
1960	2-Amino-3-thioacetylenglycol-1,4-naphthoquinone		3/3	—	—
1989	2-Hydroxy-3-thioacetylenglycol-1,4-naphthoquinone	144~147	—	—	—
1921	2 (2'-Carboxyphenylthio)-1,4-naphthoquinone	198~200	—	—	—
1918	2-(2'-Carboxyphenylthio)-1,4-naphthoquinene-sodium-bisulfite.		—	—	—
1905	2-Methylthio-1,4-naphthoquinone-sodium-bisulfite		—	—	—
2010	2-Ethylthio-1,4-naphthoquinone oxim	87	—	—	—

Weekly body weight changes and the number of days of survival were used as criteria in judging the therapeutic effect, always with due attention to the state of abdominal distension and general physical conditions of the mice. Microscopical examination of ascites was not made except in the case of necessity in establishing diagnosis.

Results

Tables 1 and 2 show the details of *in vitro* tests, and Tables 3 and 4 those of *in vivo* experiments.

In Tables 1 and 2, the results are expressed in the term of the number of

Table 2. Effect of Other Quinone Derivatives on the Survival *in vitro* of NF mouse sarcoma

Compounds			Tumoricidal effect at		
No.		M. p.	0.05%	0.01%	0.005%
1912	1, 4-Naphthoquinone	125° C	3/3	—	—
296	1, 2-Naphthoquinone	115～120	3/3	3/3	—
1913	2-Methyl-1, 4-naphthoquinone	106	4/4	3/4	3/4
1956	2-Methyl-1, 4-naphtoquinone diacetate	113	3/3	3/3	—
199	P-Xyloquinone	125	5/5	4/5	—
1906	2-Methyl-1, 4-naphthoquinone-sodium-bisulfite		—	—	—
1981	2-Methyl-3-hydroxy-1, 4-naphthoquinone	172	—	—	—
1966	2, 3-Dichloro-1, 4-naphthoquinone	189	—	—	—
1876	2-Chloro-3-hydroxy-1, 4-naphthoquinone	196	2/4	—	—
1950	2-Chloro-3-Amino-1, 4-naphthoquinone	199	—	—	—
1875	2-Chloro-3-ethoxy-1, 4-naphthoquinone		5/5	—	—
1895	2, 5-Diamino-1, 4-naphthoquinone	150～155	3/3	—	—
1904	3, 8-Bischloroacetylamino-1, 4-naphthoquinone	>270	4/4	—	—
1896	1-Oxy-4-amino-naphthalene-2, 5-diamino hydrochloride	>240	5/5	5/5	3/5
1954	Potassium-1, 4-naphthoquinone-2-sulfonate		—	—	—
1988	2-Dimethylamino-1, 4-naphthoquinone	213～216	—	—	—
1991	1, 4-Naphthoquinone oxim	190	3/3	3/3	2/3
1997	1, 4-Naphthoquinone dioxim	207	1/3	—	—
2016	1, 2-Naphthoquinone oxim	155	1/2	1/2	—
2018	1, 2-Naphthoquinone dioxim	178	—	—	—
441	o-Carboxybenzene xyloquinone imide	118～120	3/3	1/3	
447	Xyloquinone thiourea	154～158	—	—	—
1910	Benzoquinone-mono-semicarbazone	170	2/3	—	—
53	anthraquinone	286	—	—	—
1034	1, 2, 3, 5, 6, 7-Hexahydroxy-anthraquinone		—	—	—
182	2, 3, 5-Trihydroxyanthraquinone		3/3	—	—
1195	3-Hydroxymethyl-anthraquinone-1-arabinoside	101～103	—	—	—

Table 3. Effect of Selected Quinone Derivatives on Ehrlich Ascites Carcinoma *In Vivo*.

No.	Compounds	Treated Groups				Control groups			Difference in survival days	Lowest effective concent. *in vitro* tests
		Doses mg/kg	No. of mice	Average body wt. change after 1 week	Average survival days	No. of mice	Average body wt. change after 1 week	Average survival days		
1883	2-Methylthio-1, 4-naphthoquinone	15	5	−1.0	17.8	4	+1.6	12.5	+5.3	0.005
1883	2-Methylthio-1, 4-naphthoquinone	5	4	+1.2	19.0	4	+3.5	13.0	+6	0.005
1886	2, 3-Dimethylthio-1, 4-naphthoquinone	20	5	−2.8	17.8	2	−0.4	9.5	+8.3	0.005
1886	2, 3-Dimethylthio-1, 4-naphthoquinone	10	5	−0.7	15	2	−0.4	9.5	+5.5	0.005
1902	2-Carboxy-methylthio-1, 4-naphthoquinone	10	3	+1.1	12.7	4	+3.6	13.5	−0.8	0.01
1920	2-Thioacetylenglycol-1, 4-naphthoquinone	15	3	−1.8	19.0	5	+5.3	9.6	+9.4	0.01
1929	2-Methylthio-1, 4-naphthoquinone diacetate	10	4	+0.1	12.5	4	+2.2	16.5	+4.0	0.01
476	2-Ethylthio-1, 4-naphthoquinone	10	5	−1.08	19.8	4	+2.2	16.5	+3.3	0.01
1913	2-Methyl-1, 4-naphthoquinone	10	5	+2.5	11.2	4	+1.6	12.5	+1.3	0.005
1896	1-Oxy-4-imino-naphthalene-2, 5-diamino hydrochloride	10	5	+0.7	12.4	2	+0.6	17.0	−4.6	0.01
1991	1, 4-Naphthoquinone oxim	10	4	+1.6	16	2	+0.6	17.0	−1.0	0.005

Table 4. Effect of Selected Thio-naphthoquinone Derivatives on Ehrlich Ascites Carcinoma *In Vivo*.

No.	Compounds	Treated Groups								Control Groups				
		Doses	Mouse No.	Initial body weight g	Body weight after 1 week	Difference	Body weight after 2 weeks	Difference	Survival days	Mouse No.	Initial body weight	Body weight after 1 week	Difference	Survival days
1883	2-Methylthio-1,4-naphthoquinone	15 mg/kg	1	14	12.5	−2.5	15.0	+1.0	28	1	13	17.1	+4.1	14
			2	14	14.8	+0.8	14.6	+0.6	16	2	13			(6)
			3	14	13.3	−0.7	14.0	0	16	3	13	14.6	+1.6	11
			4	14	13.6	−0.4			13	4	13	13.5	+0.5	11
			5	14	11.7	−2.3	14.8	+0.8	16	5	13	13.3	+0.3	14
	(Average)					−1.0		+0.6	17.8				+1.6	12.5
1886	2,3-Dimethylthio-1,4-naphtoquinone	20 mg/kg	1	15.9	12.0	−3.9			13	1	16.0			(5)
			2	13.0	10.7	−2.3			8	2	15.9	15.5	−0.4	(24)
			3	13.4	11.8	−1.6	15.7	+2.3	27	3	13.5			(6)
			4	13.4	10.5	−2.9	11.7	−1.7	29	4	12.0	11.0	−1.0	11
			5	14.9	11.3	−3.6			12	5	13.2	13.0		8
	(Average)					−2.8		+0.3	17.8					9.5
1920	2-Thioacetylenglycol-1,4-naphthoquinone	15 mg/kg	1	14					(5)	1	14	18.8	+4.8	10
			2	14	13.4	−0.6			10	2	12	16.5	+4.5	9
			3	14	11.6	−2.4			17	3	12	18.2	+6.2	9
			4	14					(4)	4	14	20.6	+6.6	11
			5	14	11.4	−2.6			30	5	14	18.6	+4.6	9
	(Average)					−1.8			19.0				+5.3	9.6

negatives over the total number of implants made for each dilution of test substances. For example, 3/3 means that all the three implants resulted in no tumor growth; 1/3, only one out of three implants resulted in negative, i. e., two tumors obtained from three implants.

The cases where all implants produced tumors are indicated by minus signs (−), meaning that there was no effect.

Tables 3 and 4 require no explanation. It only needs to be stated that mice which died of intoxication while still under treatment are disregarded in calculating the average survival days. A single case of natural immunity to Ehrlich carcinoma was encountered among the control mice, and this case was likewise disregarded.

Discussion

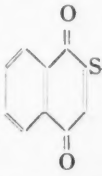
The data presented in Table 1 show that 2-methylthio-1, 4-naphthoquinone and dimethylthio-1, 4-naphthoquinone, produced by the introduction of methylthio radical in position 2 and in positions 2 and 3, respectively, of 1, 4-naphthoquinone, are capable of killing NF sarcoma cells at the concentration as low as 0.005 %. The derivatives in which ethylthio, carboxymethylthio or thioacetylenglycol radical is introduced at position 2 are next active, showing tumoricidal effect at 0.01–0.005 % concentrations. Among the 1, 2-naphthoquinone derivatives other than thioquinones, 2-methyl-1, 4-naphthoquinone, 1-oxy-4-imino-naphthalene-2, 5-diamino-hydrochloride and 1, 4-naphthoquinone oxim are effective up to 0.005 %. These effective quinones become less effective when they are changed to diacetates, and the simultaneous introduction of other "effective" radicals not only fails to increase the effectiveness but acutally decreases it. Derivatives of 1, 4-naphthoquinone with hydroxy, chlorine, carboxymethylthio, amino, sulfon, dimethylamino, or ethoxy radicals at positions 2 and 3 are practically without effect. A few anthraquinone derivatives we tested are likewise without effect, although previous authors reported some slight effect for certain other derivatives of this group.

Effect *in vivo* on Ehrlich ascites carcinoma was perceptible in the cases of 2-methylthio-1, 4-naphthoquinone (1883), 2, 3-dimethylthio-1, 4-naphthoquinone (1886), 2-thioacethylenglycol-1, 4-naphthoquinone (1920) and 2-ethylthio-1, 4-naphthoquinone (476). Treatment with these thio-quinones produced a significant prolongation of survival days. It is interesting to note that other quinones were quite ineffective in *in vivo* tests with Ehrlich ascites carcinoma, even those that were found to be effective at the concentration of 0.005 % in *in vitro* tests.

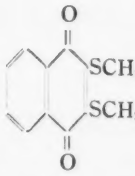
As may be seen from Table 4, daily intraperitoneal injections of 10–20 mg/kg for 5 days tended to reduce the body weight of injected mice at the end of one week, and two mice died of intoxication during the injection period with

2-thioacetylenglycol-1, 4-naphthoquinone. The toxicity for mice in the term of LD_{50}, as determined in separate experiments, was 100 mg/kg for 2-methylthio-1, 4-naphthoquinone, and less that 10 mg/kg for 2-thioacetylenglycol-1, 4-naphthoquinone.

The stuctures of thio-quinones we found to be effective against Ehrlich carcinoma are:



R=CH_3– (1883)
=CH_3CH_2– (476)
=$CH_2OH \cdot CHOH$– (1920)



(1886)

It may be noted that these are all derived by the introduction of alkylthio radical at position 2 of quinone. Increase in the number of C of the alkylthio radical lowers the solubility of the compound in water.

Summary

By testing a long series of quinone derivatives for their tumoricidal action on NF sarcoma *in vitro*, and for their inhibiting action on Ehrlich ascites carcinoma *in vivo*, it was demonstrated that certain thio-quinone derivatives show significant anti-cancer effects.

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Postscript:

After writing the foregoing, our attention was called to the recent important paper by Domagk, Petersen and Gauss, "Ein Beitrag zur experimentelle Chemotherapie der Geschwülste", Zeitschrift für Krebsforschung, Bd. 59, S. 617, 1954. Using Yoshida rat sarcoma transplanted intramuscularly, and starting treatment 24 hours after transplantation, these authors reported a complete suppression of tumors by 2, 5-bisethlene-imino-1, 4-benzoquinone. Effect of this quinone derivative on Ehrlich carcinoma was very slight.

This work of Domagk and collaborators affords additional emphasis on quinone derivatives in experimental chemotherapy of cancer.

要　旨

キノン類の制癌作用について

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我々は著者の一人福岡による簡単，かつ実用性のある制癌性化合物を選択する方法を用いてキノン類の制癌作用を選別した。試験に供した化合物は主として 1, 4-naphthoquinone の誘導体であるが，その内数種を除いては制癌作用に関する文献に記載されていないものである。この方法で有効であった化合物に就いては広く用いられている Ehrlich 腹水癌に対する in vivo の制癌作用をも検査した。

その結果，われわれは 1, 4-naphthoquinone の alkylthio 誘導体が制癌性を有することを見出すことができた。すなわち，2-methylthio-1, 4-naphthoquinone, 2, 3-dimethylthio-1, 4-naphthoquinone, 2-thioacetylenglycol-1, 4-naphthoquinone, および 2-ethylthio-1, 4-naphthoquinone 等の thioquinone である。

(一部分文部省科学研究費による)

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THE ROLE OF THIOL GROUPS IN THE MECHANISM OF RADIATION INJURY AND PROTECTION AGAINST IT

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As remarked by Weiss (1), ionizing radiations are believed to decompose water molecules into free hydrogen atoms and hydroxyl radicals, and in the presence of dissolved oxygen, the free hydrogen atoms produce powerful oxidizing free radicals, i.e., HO_2 which in turn are degraded to H_2O_2. Barron *et al.* (2) demonstrated that -SH enzymes are quite sensitive to oxidation when irradiated in dilute aqueous solution and it is possible to protect and to reactivate them *in vitro* by addition of GSH. In this connection, they demonstrated that ionizing radiation can oxidize -SH compounds (3). Furthermore, with total body irradiation of rats Barron found that the metabolism of various substances requiring -SH enzymes were diminished immediately after irradiation, with thymus as the only exception (4). These results would furnish an adequate explanation for the effects of radiation, but unfortunately confirmation seems to have been lacking and indeed some workers (5, 6) have reported no inhibition of -SH enzymes with lethal doses of X-irradiation.

On the other hand, an important clue to the mechanism of radiation injury may be disclosed by the studies on the means of protection against it. On this line Patt *et al.* (7) reported that -SH compounds, i. e., cysteine and GSH, are effective for radiation protection and especially cysteinamine has been found by the other workers (26) to be most effective.

The present experiments were undertaken to evaluate the effect of X-irradiation on the -SH content of isolated mitochondria and its enzymatic activities requiring -SH groups (succinoxidase and phosphorylation activities coupled to the oxidation of succinate or β-hydroxybutyrate as well as adenonsinetriphosphatase (ATP-ase) activity). At the same time, in order to elucidate the role of -SH groups in the mechanism of chromosome aberration induced by irradiation, radiation effects on the -SH content and viscosity of isolated chromosome preparation were examined. The work was extended to protection experiments with thiosulfate and taurine in comparison with the effect of cysteine, for whole body irradiation of rats.

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Experimental

Irradiation procedures[1]—For *in vitro* X-irradiation, the physical factors were 140 kv. and 3 mA., using 0.1 mm. copper and 1 mm. aluminium added filter. Target distance was 25 cm. and total dose up to 3000 r at a rate of 48 r per minute. The animals used in the protection experiments were rats weighing about 100g., and they were given a total exposure of 600 r, which has been shown to be a LD_{50} dose in some laboratories (8, 9).

Preparation of subcellular particle fraction.—Rat liver mitochondrial fractions were prepared by isotonic sucrose procedure as described by Schneider (10) and hypertonic sucrose procedure as employed in the previous experiments (11). It was confirmed again that the latter procedure gives a preparation containing no freely reacting -SH groups as revealed by amperometric titration (12) and ferricyanide method (13).

Thymus chromosomes were prepared from young pig as well as calf, by the method of Mirsky and Ris (14). Their chemical compositions and microscopic and electronmicroscopic figures were very similar to those of Mirsky and consisted of DNA-histone, free from RNA, so that there was no doubt that they came from nuclei exclusively.

Enzyme assay.—The assay for the succinoxidase activity of mitochondria was carried out as described in the previous report (11). It was found that the liver mitochondria is poor in creatine phosphokinase activity (15) and consequently it was impossible to trap off the high energy phosphate ester as creatine phosphate. Therefore, to assay the phosphorylation activity coupled with the oxidation of succinate or β-hydroxybutyrate[2] creatine phosphokinase was added in the system besides creatine. The latter enzyme was prepared as descrived by Askonas (16), that is, extracted with water from rabbit muscle and fractionated by acetone at low temperature. The individual components of the assay medium for succinate oxidation system are described in Table III, and in Table IV for β-hydroxybutyrate system.

Mg-activated ATP-ase activity of isolated mitochondria preparation was determined in the system described in Table V.

Determination methods.—The total -SH contents of mitochondria, chromosome as well as liver and spleen were determined by amperometric titration in the presence of ethyl alcohol as described in the previous paper (11).

Blood GSH was determined by the same method after hemolysis with saponin and sulphosalicylic acid deproteinization of blood. Ribonucleic acid (RNA) and

1) The writer wishes to express his graditude to the staff of the Department of Radiology of the University of Niigata Medical School for their kind help in this procedure.

2) Sodium-dl-β-hydroxybutyrate was prepared by deaminating 1-β-aminobutyrate according to Fisher and Scheibler (17).

desoxyribonucleic acid (DNA) contents of liver and spleen were assayed by ultraviolet absorption (at 260 mμ) of $HClO_4$ fractions of 10 per cent water homogenates, modified in some points, according to the procedure of Ogur and Rosen (18).

Results

I. The Role of -SH Groups in The Biological Process Following X-irradiation.

i) Radiation Effect on -SH Content and Succinoxidase Activity of Isolated Liver Mitochondria.

At the first attempt, the mitochondrial preparation, soon after its preparation, was exposed to different X-ray doses up to 1000 r in an ice bath, but the radiation effects were found to be rather slight, showing 8 per cent reduction in -SH content and corresponding inactivation of succinoxidase activity. In this experiment, however, the correlation between X-ray dose and the effect could be demonstrated. Then the dose was raised to as high as 3000 r and dissolved gases in the suspension were replabed by pure O_2 or N_2 to clarify the influences of dissolved gasses. The results in these conditions are given in Table 1. It was confirmed that removal of O_2 during the irradiation abolished the reduction of -SH content and enzymatic activity almost completely, but the exposure in the presence of O_2 the reduction rate of -SH content was 15 per cent, with the corresponding inactivation rate of succinoxidase activity.

Table 1. Radiation Effects on -SH Content and Succinoxidase Activity of Mitochondria.

Mitochondria preparation method	Replaced gas	O_2		N_2	
	X-irradiation	3000 r	0	3000 r	0
Isotonic sucrose procedure	O_2 uptake μl. per 5 minutes.	33.5	40.5	41.0	41.2
	Inhibition rate in o/o*	17.3	—	0.5	—
	-SH content μM./0.2 ml. of mitochondria	0.092	0.106	0.106	0.105
	Decreased rate in o/o*	13.2	—	(+1.0)	—
Hypertonic sucrose procedure	O_2 uptake μl. per 5 minutes.	40.5	43.7	42.6	44.5
	Inhibition rate in o/o*	7.5	—	3.3	—
	-SH content μM/0.2 ml. of mitochondria	0.132	0.144	0.146	0.155
	Decreased rate in o/o*	8.5	—	5.8	—

* The values of no irradiation groups are used as control. The reaction mixture of enzyme assay; Water, 0.5 ml.; 0.1 phosphate buffer (pH 7.4), 1.0 ml.; 0.5 M succinate, 0.30 ml.; 1×10^{-4} M cytochrome c, 0.40 ml.; 4×10^{-3}M $CaCl_2$, 0.30 ml.; 4×10^{-3}M $AlCl_3$, 0.30 ml., and mitochondria suspension 0.2 ml. (total volume 3.00 ml.). Temperature 38°C.

Table 2. Reactivation of Succinoxidase by GSH.

Replaced gas	O_2		N_2		Control (no irradiation)
GSH 5 mg.	−	+	−	+	
O_2 uptake μl. per 5 minutes.	29.6	32.6	33.0	33.2	34.1
Activity in per cent	86.8	96.0	97.0	97.5	100
-SH content μM per 0.5 ml. of mitochondria	0.324		0.340		0.340

The doses of X-irradiation were equally 3000 r, and the mitochondria suspension was prepared by the isotonic sucrose procedure. The succinoxidase was assayed by the same system as described in Table 1.

In the reactivation experiments, 5 mg. of GSH per each Warburg's flask of succinoxidase assay was added to mitochondria, which had been irradiated with 3000 r in the presence of O_2. As shown in Table 2, reactivation of the enzymatic activity was nearly complete.

ii) Radiation Effect on Aerobic Phosphorylation.

As demonstrated in the preceding section, the effect of radiation on succinoxidase was too slight to account for the serious *in vivo* effect of relatively high doses such as 3000 r. Experiments were, therefore, extended to aerobic phosphorylation to seek for more drastic effect on enzyme systems. In succinate system, as shown in Table 3, creatine phosphokinase preparation was essential for trapping phosphate ester and GSH acceralated phosphorylation appreciable more than oxidation, consequently raising the P/O ratio. Then, in order to examine the radiation effect on phosphorylation GSH was omitted from the system, and for the reactivation of its efficiency GSH was added to the system.

The results were shown in Fig. 1. By irradiation of 3000 r dose, the ability of the liver mitochondria to esterify P is so strongly imparied, that the P/O ratio decreased from 0.81 to 0.29 at incubation time of 15 minutes. But in the presence of GSH the ability was completely restored; P/O ratio for the control was 0.93 and for the irradiated 0.90.

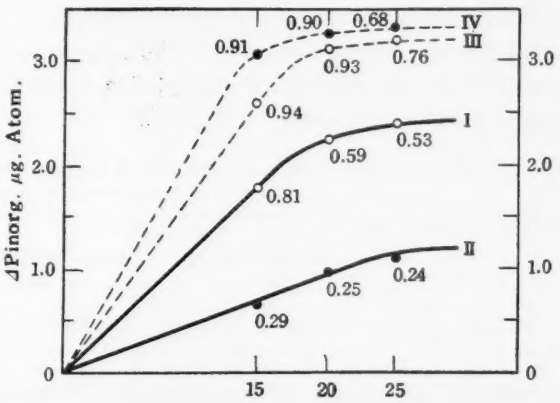
Since the succinoxidase system dose not depend on DPN, phosphorylation coupled with β-hydroxybutyrate oxidation was tested following succinate system. It is known that rat liver mitochondria is devoid of the ability to oxidize acetoacetate, so by using β-hydroxybutyrate as substrate one can see one step oxidation to acetoacetate and phosphorylation coupled with DPNH oxidation. As shown in Table 4 X-irradiation imparied the phosphorolation, although the degree of the impairment was slighter than in succinate system, and had no influence on the oxidation of β-hydroxybutyrate, the P/O ratio being lowered from 1.35 to 0.91.

Table 3. Effect of Creatinephosphokinase (Cp- kinase) and GSH on Oxidative Phosphorylation in Succinate System.

	Start	Complete system	Component omitted			
			GSH	Succ. GSH	Cp-kinase	GSH Cp-kinase
μg. Atom O_2 in 20 min.	—	7.5	5.4	0	7.18	5.6
True inorg. P. μg.	336	126	266	462	329	336
ΔP inorg. P. μg. Atom.	—	6.78	2.26	(+4.0)	0.266	0
P/O	—	0.89	0.41	—	0.037	0
Phosphocreatine P μg.	21	210	91	77	35	14

The complete assay system contained creatine 30 mg. in water 1.2 ml.; mixture of 0.33 M KCl, 0.02 M phospate buffer (pH 7.4) and 0.09 M NaF, 0.5 ml.; 0.02 M ATP, 0.2 ml.; cytochrome c 1×10^{-4} M, 0.4 ml.; mixture of 0.09 M succinate and 0.10 M $MgCl_2$, 0.2 ml.; 0.1 M GSH, 0.2 ml.; Cp-kinase preparation 0.05 ml. and mitochondria suspension 0.25 ml. (total volume 3.00 ml.) Temperature 25°C.

Fig. 1. Radiation Effect on Oxidative Phosphorylation in Succinate System and Reactivation with GSH.



Figures in the graph represent the P/O ratios at each time of each experiment.

Experimental conditions were the same as described in Table 3. Curves 1, 3, for the control of no irradiation; Curves 2, 4, for the irradiated one (3000 r). From the reaction mixture of Curves 1, 2, GSH was omitted; that of Curves 3 and 4 was the complete system in Table 3, in which GSH was added.

iii) Radiation Effect on ATP-ase Activity.

In connection with phosphorylation experiments, ATP-ase activity of mitochondria was examined. Mg-activated ATP-ase activity was determined under conditions comparable to those in phosphorylation experiment, except that F^-ion

Table 4. Radiation Effect on Oxidative Phosporylation in β-hydroxybutyrate System.

	No irradiation			X-irradiated (2770 r)		
Incubation time in min.	0	20	30	0	20	30
True inorg. P μg.	329	301	282	339	315	308
⊿P inorg. μg. Atom.	1.35*	2.26	2.71	1.02*	1.80	2.03
O_2 uptake μg. Atom.	(1.00)	2.01	2.98	(1.12)	2.24	3.18
P/O	(1.35)	0.75	0.55	(0.95)	0.68	0.49

* Calculated as the difference between the true inorganic P values of before equilibrium (371 μg.) and that of after equilibrium.

The values in ( ) were obtained by extrapolation from the values of 0–20 interval.

The assay system contained 0.3 M glycylglycine buffer (pH 7.4) 0.2 ml.; cytochrome c 1×10^{-4}, 0.3 ml.; 1% DPN 0.4 ml.; mixture of 0.09 M β-hydroxybutyrate and 0.1 M $MgCl_2$, 0.3 ml.; 0.02 M ATP, 0.4 ml.; mixture of 0.33 M KCl, 0.02 M K-phosphate (pH 7.4) and 0.09 M NaF, 0.5 ml.; Cp-kinase preparation 0.05 ml.; water, 0.45 ml.; mitochondria suspension 0.4 ml. (total volume 3.00 ml.) Incubation temperature was 25°C, and the equilibrium period was 10 minutes.

was omitted, and the details components are presented in Table 5. As shown in the table, ATP-ase activity was directly proportional to enzyme concentration, as expressed on the basis of the amount of N in the enzyme used. In contrast to the case of succinoxidase and oxidative phosporylation, X-irradiation increased the ATP-ase activity about 20 per cent.

Table 5. Radiation Effect on ATP-ase Activity of Mitochondria.

Enzyme volume	3000 r X-irradiated	No irradiation.
ml.	liberated P μg.	liberated P μg.
0.15	55	42.5
0.30	104.8	82.5
Liberated P μM/N mg.	4.33	3.55

The reaction mixture of Mg^{++} activated ATP-ase contained 0.3 M glycylglycine buffer, 0.25 ml.; 0.025 M $MgCl_2$, 0.2 ml.; 0.15 M KCl, 0.15 ml., only in the case of mitochondria volume 0.15 ml.; 0.02 M ATP, 0.25 ml., then mitochondria suspension in 0.15 M KCl, 0.15 ml. or 0.30 ml. (total volume 1.00 ml.) Incubation period 10 minutes, incubation temperature 30°C.

iv) Radiation Effects on the -SH Content and Viscosity of Isolated Chromosomes.

The chromosome preparation which was obtained from young pigs thymus glands 150 g. (pool from four pigs) and suspended in 250 cc. of saline, and that from 100 g. calf thymus in 100 cc. of saline were X-irradiated up to 3000 r dose under the atmosphere of air of N_2 (Table 6). Pig thymus chromosome contained

-SH groups 0.238 per cent of its histone moiety calculated as cysteine, which was 1.6 times much as that in calf thymus (0.145 per cent).

In both preparations, X-irradiation did decrease their -SH contents, especially intensely in the case of pig thymus chromosome; reduction amounting to 42 per cent (from 0.238 to 0.138 per cent). at 3000 r dose. In contrast to the case of irradiation in the presence of air, -SH content was firmly preserved in the absence of O_2 (replaced with N_2) even after exposure to the highest dose.

In respect to irradiation effects on the viscosity of chromosome solution, which was made of equal volume of chromosomes suspension in 0.14 M NaCl and 0.1 M NaOH solution, there was only slight decrese of viscosity independently of the presence or absence of O_2 and 0.01 M cysteine (in the final concentration) during exposure to X-ray.

Table 6. Radiation Effect on -SH Content of Isolated Thymus Chromosome.

i) Chromosome preparation from pig thymus

X-ray dose r / Dissolved gas	3000	1500	0
N_2	0.232 (97)		
Air	0.138 (58)	0.194 (82)	0.238 (100)

ii) Chromosome preparation from calf thymus

X-ray dose r / Dissolved gas	3000	2000	0
N_2	0.142 (98)		
Air	0.114 (78.3)	0.140 (87)	0.145 (100)

All figures represent the cysteine content in per cent of their histone moieties. Their residual per cent are presented in parentheses.

II. The Role of -SH Groups in the Protection Against Radiation.

Protection actions of chemicals against irradiation have been determined mainly by the modification in survival rate, body weight loss and hematological changes, as adopted by Patt *et al.* in the case of cysteine (19) and GSH (20). But for the purpose of pursuing the mechanism of irradiation injury and of protection, chemical changes, i.e., total -SH grougp as well as RNA and DNA contents of liver and spleen were examined besides hematological changes.

The rats were divided into 6 groups as described in Table 7, that is; 1) Control group of no irradiation and treatment, 2) Group of no irradiation but with thiosulfate treatment, 0.5 g./kg., 3) Group treated with saline and irradiated with 600 r dose, 4) Group treated with thiosulfate, 0.5 g./kg., then X-irradiated,

5) Group treated with cysteine (pH 7.0), 0.18 g./kg., then X-irradiated, and 6) Group treated with taurine, 0.25 g./kg., and X-irradiated. Each group consisted of 4 or more animals. Individual chemicals were injected peritoneally, just before the irradiation of 600 r dose, with the exception of taurine, which was injected 1 hour before irradiation. The animals were killed 48 hours after

Table 7

1) Radiation Effects on Tissue and on Body Weight.

	Treatment	Total -SH cont. mM/100 g. tissue		Nucleic acid P mg./100 g.				Body weight diff. g.
				Spleen		Liver		
		Spleen	Liver	RNA	DNA	RNA	DNA	
No irradiation	No treatment	1.25 (±0.07)	2.02	105.6	177.5	97.4	41.0	+13
	Thiosulfate 0.5 g./kg.	1.46 (±0.15)	2.07	97.4	208.3	79.5	42.5	
X-irradiation 600 r	Saline as control	1.02 (±0.05)	2.24	50.5	59.6	99.1	51.4	−11
	Thiosulfate 0.5 g./kg.	1.30 (±0.07)	2.16	62.7	77.4	99.1	49.3	−2.3
	Cysteine 0.18 g./kg.	1.26 (±0.04)	2.19	62.3	74.2	101.2	51.8	−2.7
	Taurine 0.25 g./kg.	1.25 (±0.06)	2.20	58.5	121.0	98.0	45.1	−7.3

2) Radiation Effect on Blood.

	Treatment	GSH mg./dl.	Hb g./dl.	Red cell count million/mm³	White cell count per mm³	
					Lympho.	Polymor.
No irradiation	No treatment	27.4	9.95	6.80	3015	2565
	Thiosulfate 0.5 g./kg.			6.37	3010	1223
X-irradiation 600 r	Saline as control	28.9	9.98	6.67	465	758
	Thiosulfate 0.5 g./kg.	33.1	10.2	6.01	427	1248
	Cysteine 0.18 g./kg.	32.0	9.89	6.47	646	1106
	Taurine 0.25 g./kg.	31.2	9.88	7.00	1966	

All figures are the means of each group consisted of 4 or more rats of about 100 g. weight.

The animals were killed 48 hours after irradiation for the determination of radiation effects.

irradiation, with appropriate controls, for the above stated determinations. The results are summarized in Table 7.

i) Effect on -SH Levels of Spleen and Liver.

Total -SH levels of spleen decreased slightly but significantly by the total body irradiation of 600 r, 10 per cent in average (from 1.25 mM/100 g.), and this decrease could be prevented satisfactorily by the pretreatment with thiosulfate, and to a lesser extent by cysteine and taurine. In contrast to the case of spleen which is believed to be sensitive to irradiation, X-ray somewhat increased the -SH level in liver, and there was shown no modification by the pretreatment with these chemicals.

ii) Effect on Nucleic Acid Contents.

As one can see in Table 7, there was a sharp drop in RNA and DNA concentration of spleen after irradiation, and this was a particularly marked difference from what occurred in liver. Without pretreatment the concentration of RNA and DNA of spleen was lowered to about 2/3 and 1/2, respectively, of the control values by irradiation. Cysteine and thiosulfate could prevent these decreases to some extent, and it was noteworthy that taurine had a lesser effect on RNA but excellent on DNA, so as to be better than the effect of cysteine and thiosulfate.

iii) Effects on Hematological Changes and Body Weight Loss.

As the time interval between irradiation and determination was so short as 48 hours, there was no remarkable changes in red blood cells count, hemoglobin content as well as GSH in whole blood. In contrast, white blood cells, especially lymphocytes, were revealed to be most sensitive to irradiation. Cysteine, thiosulfate and equally exhibited significant protection against the decrease of white blood cells, more selectively for polymorphonuclear leucocytes, than for lymphocytes.

Body weight loss in average by irradiation was 11 g., while that of non-irradiated group increased 13 g. during the same period. Cysteine and thiosulfate could reduce the loss to 2–3 g., while taurine only to 7.3 g.

Discussion

By irradiation *in vitro* of isolated mitochondria, it was confirmed that ionizing radiation, oxidizing the -SH groups in the presence of O_2, inhibited the succinoxidase activity, although the inhibition rate was very slight considering the relatively high dose. But this was predicted from the previous experiments (11), in which the relative high resistance of succinoxidase of mitochondria to oxidizing agents compared to mercaptide-forming one was demonstrated, due to containing no freely reacting -SH groups. It was very regretable that due to technical difficulties experiments could not be carried out with spleen mitochondria, which

is in fact more sensitive to irradiation.

The observation of Fisler *et al.* (21), that only spleen succinoxidase activity was reduced and protected by cystein pretreatment, among the enzyme systems tested in liver, spleen and kidney after whole body irradiation, would be explained by the above stated results and by the fact that spleen total -SH groups diminished after whole body irradiation and was preserved by cysteine pretreatment.

As to the mechanism of radiation injuries, one should atach more importance to the decoupling of oxidation and phosphorylation than to oxidation itself, considering the severity of inhibition rate of the former, as revealed in the succinate system. In the case of β-hydroxybutyrate system, the relatively slight impairment of phosphorylation activity might be due to the ATP-ase activating action of radiation on mitochondria. In this connection, it must be recalled that according to Barron's studies, ATP-ase was the most sensitive enzyme to radiation in aqueous solution. But this was not the case in mitochondria. Mitochondria preparation isolated by mild procedure, as in our experiments, contains ATP-ase only in its inactive form and the enzyme is activated by deteriorating process, as observed by Killey (22). So X-ray might act as one kind of degenerating factors on mitochondria, thus activating its ATP-ase activity. Explanation for the severe impairment of phosphorylation in succinate system is obscure, but the complete reactivation by GSH may suggest the necessity of highly active -SH groups in phosphorylation. This was previously demonstrated in Hirade's experiment (23), in which he observed that -SH blocking agents inactivated phosphorylation stronger than oxidation in succinate system.

It was reported by Maxwell *et al.* (24) that after whole body irradiation, the oxidative phosphorylation activity of spleen mitochondria in presence of succinate was impaired remarkably, but the clear explanation for the reason was lacking. According to our opinion, this might be caused by the reduction of -SH groups in spleen mitochondria, as demonstrated in the *in vivo* experiments.

It is of interest that the -SH groups of chromosome was essentially more sensitive to oxidation by X-irradiation than those of mitochondria. Chromosome contains little -SH groups, 0.238 per cent in pigs thymus, and 0.145 per cent in calf thymus of their histone moieties, while -SH groups of liver mitochondria amount to over 0.7 per cent of its total dry weight as revealed by previous experiments (11). Thus Barron's assumption (25) for the mechanism of radiation induced mutation, that chromosomes possess few -SH groups in their molecules, and on oxidation by X-irradiation there would be molecular aggregation by binding of two molecules through -S-S- linkage, was supported by this experiment. The inhibition of chromosome aberration by irradiation in the absence

of oxygen (27) is consistent with the author's finding that -SH groups in chromosome are preserved firmly in their conditions.

In protection experiments, thiosulfate and taurine were employed in comparison with cysteine, because thiosulfate was revealed active for protection against, and treatment of many intoxications as -SH compound (constitution of free $H_2S_2O_3$ is believed to be $HS\text{-}SO_3H$), and taurine, although it contains no -SH group, it was demonstrated to preserve GSH levels of blood in such cases as alloxan diabetes or phenylhydrazine anemia[3]. In this experiment thiosulfate was found to be more effective than cysteine in various points, i. e., in maintaining total -SH and both nucleic acid contents of spleen as well as the body weight; and taurine showed a specific effect in preventing spleen DNA decrease.

Summary

1) By X-irradiation *in vitro* in the presence of O_2, the -SH content of isolated liver mitochondria was oxidized and succinoxidase activity was inhibited slightly in accordance with the extent of oxidation of the -SH content, but was reactivation by GSH.

2) X-irradiation impaired the oxidative phosphorylation activity of isolated mitochondria strongly in succinate system; lesser in β-hydroxybutyrate system, and in the former case GSH could reactivate phosphorylation ability almost completely. In contrast, ATP-ase activity of mitochondria was activated by irradiation. The inconsistancy with Barron's results was discussed.

3) The -SH content of chromosome preparation decreased after irradiation only in the presence of O_2, and the significance of this result in relation to chromosome aberration was discussed.

4) The protective effect of thiosulfate and taurine for whole body irradiation of rats were examined in comparison with that of cysteine, and the former was found to be effective in protecting -SH content of spleen and the latter most excellent for preserving spleen DNA contents.

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要　旨

放射線障害並びにその予防における SH 基の役割について

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SH 酵素が水溶液中で放射線照射により非活化をうけることは Barron 等によって認みられているが，これが果して放射線障害の機序となりえるか否かにはまだ疑問がある．

本実験ではまず，鼠肝よりのミトコンドリヤに X 線を照射し SH 基の減少と酵素活性の低を追求した．3000 r の照射で酸素の溶在するときにのみ軽度の SH 基の減少とそれに匹敵するコハク酸酸化酵素能の低下を認めた．また同酵素能は GSH の添加により再賦活された．

さらに高度の阻害は酸化に共軛する燐酸エステル化能にあらわれ，P/O 比はコハク酸を基質とした場合 0.81 から 0.29 に減じ，GSH によりほぼ非照射対照値に恢復した．

次に豚および小牛の胸腺から Chromosome を分離し X 線を照射したところ，その SH 基はミトコンドリヤ中のものより敏感で豚胸腺よりの標本は減少度 40% にもおよんだ．

さらに鼠の全身照射 (600 r) による脾臓 SH 基，RNA，DNA 量の低下および体重の減少がチオ硫酸ソーダによって SH 化合物システインと同様にかなり予防されることが示された．

以上の知見は放射線障害の一機序として SH 基侵襲を考える根拠となり，また SH 化合物による障害予防のうらづけをなすと考えられる．

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ERRATA

In Gann, Vol. 45, No. 4, December 1954, p. 646, charts above the explanations of Fig. 2 and Fig. 3 should be transposed.

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